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# Determinants of local calcium signaling in retinal amacrine cells

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# DETERMINANTS OF LOCAL CALCIUM SIGNALING IN RETINAL AMACRINE CELLS

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Biological Sciences

by

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August, 2006

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# Table of Contents

Acknowledgements .....	ii
List of Tables .....	vi
List of Figures .....	vii
Abstract .....	viii
Chapter 1. Introduction .....	1
Importance of Metabotropic Glutamate Receptors in the Retina .....	2
Role of Mitochondria in Calcium Signaling in Retinal Amacrine Cells .....	4
Local Calcium Signaling in Retinal Amacrine Cells .....	7
References .....	8
Chapter 2. Immunolocalization of Metabotropic Glutamate Receptors 1 and 5 in the Synaptic Layers of the Chicken Retina .....	13
Introduction .....	14
Materials and Methods .....	15
Antibodies .....	15
Tissue Preparation for Electron Microscopy .....	16
Pre-embedding Immunoelectron Microscopy .....	17
Light Microscopy .....	18
Results .....	19
Localization of Metabotropic Glutamate Receptor 1 .....	19
Localization of Metabotropic Glutamate Receptor 5 .....	27
Localization of mGluR1 and mGluR5 to Cone Terminals .....	31
Discussion .....	31
Comparison to Results at the Light Microscopic Level .....	34
Comparison of the Localization of Group I mGluRs in Chicken and Mammalian Retinae .....	34
Presynaptic Labeling of Cone Pedicles .....	36
The Functional Significance of Group I mGluR Expression .....	36
References .....	37
Chapter 3. Local Consequences of Mitochondrial Calcium Transport in Retinal Amacrine Cells .....	42
Introduction .....	43
Materials and Methods .....	45
Cell Culture .....	46
Immunocytochemistry .....	46
Localization of Mitochondria .....	47
Calcium Imaging .....	47

pH Imaging .....	48
Ratiometric Analysis.....	48
Solutions .....	49
Results.....	49
FCCP-dependent $\text{Ca}^{2+}$ elevations in Amacrine Cells .....	50
Glutamate-dependent Calcium Elevations in Cell Bodies and Neuronal Processes .....	67
Discussion .....	72
References .....	76
Chapter 4. Conclusions .....	82
Metabotropic Glutamate Receptors and Amacrine Cells.....	83
Mitochondria and Calcium Signaling in Amacrine Cells .....	84
Local Calcium Signaling.....	86
References.....	88
Appendix: Letter of Authorization to Reprint .....	92
Vita.....	94

## **List of Tables**

2.1	mGluR1 and mGluR5 expression in the OPL of the chicken retina .....	25
2.2	mGluR1 and mGluR5 expression in the IPL of the chicken retina .....	25

## List of Figures

2.1	Controls .....	20
2.2	Localization of mGluR1 immunoreactivity in the OPL .....	22
2.3	Localization of mGluR1 immunoreactivity in the cone pedicles but not rod terminals .....	24
2.4	Localization of mGluR1 immunoreactivity in the IPL .....	26
2.5	Localization of mGluR5 immunoreactivity in the OPL .....	28
2.6	Localization of mGluR5 immunoreactivity in the IPL .....	30
2.7	SV2 expression overlaps with mGluRs 1 and 5 in cone pedicles .....	32
3.1	Distribution of mitochondria in cultured amacrine cells .....	51
3.2	FCCP-dependent $\text{Ca}^{2+}$ elevations in amacrine cells .....	52
3.3	Dependence of FCCP responses on the proximity to mitochondria .....	55
3.4	Cytosolic pH measurement and ratiometric analysis.....	55
3.5	Pre-treatment with thapsigargin alters the duration of FCCP-dependent $\text{Ca}^{2+}$ elevations .....	57
3.6	CPA alters the duration of FCCP-dependent $\text{Ca}^{2+}$ elevations in amacrine cells .....	60
3.7	Both ryanodine receptors and $\text{IP}_3$ receptors are expressed in amacrine cells .....	62
3.8	Ryanodine blocks FCCP-dependent $\text{Ca}^{2+}$ elevations .....	65
3.9	Glutamate-dependent calcium elevations in amacrine cells .....	68
3.10	Glutamate-dependent $\text{Ca}^{2+}$ elevations are locally buffered by mitochondria.....	71
3.11	Local re-release of $\text{Ca}^{2+}$ from mitochondria (mNCX) extends glutamate-dependent $\text{Ca}^{2+}$ elevations .....	73



## Abstract

Amacrine cells mediate complex lateral signaling in the inner plexiform layer of the vertebrate retina. Glutamate is the primary excitatory neurotransmitter that shapes the retinal circuitry by activating ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). One step in understanding the signaling in the inner retina is to understand the role and expression patterns of these glutamate receptors. The expression of iGluRs in amacrine cells is well documented in the literature. This study addresses the localization of Group I mGluRs in the retina. Pre-embedding immunocytochemistry combined with electron microscopy was used to study the expression of Group I mGluRs in the chicken retina. Results indicate that Group I mGluRs are expressed in the synaptic sites of the outer plexiform (OPL) and inner plexiform (IPL) layers and specifically at amacrine cell synapses. In order to understand the intricacies of amacrine cell signaling mechanisms it is important to dissect out the signaling mediated by  $\text{Ca}^{2+}$  in these cells. In this dissertation,  $\text{Ca}^{2+}$ -dependent local signaling in amacrine cells, more specifically the  $\text{Ca}^{2+}$  transport mechanisms involving mitochondria and ER, are explored. Calcium imaging experiments were performed on cultured chick amacrine cells. The results presented here suggest that there is physiological interplay between mitochondria and ER. In addition to this, it is demonstrated that ryanodine receptors are specifically involved in  $\text{Ca}^{2+}$  transport, probably via calcium-induced calcium release (CICR). Blocking the mitochondrial uniporter with FCCP or the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (mNCX) with CGP 37157 revealed that mitochondria also influence the duration of glutamate-dependent cytosolic  $\text{Ca}^{2+}$  elevations. The effects of FCCP and CGP were detectable only in amacrine cell bodies and also in regions of processes next to mitochondria, thus revealing a spatial limit to the effects of

mitochondria on cytosolic  $\text{Ca}^{2+}$ . Together, these results contribute to our understanding of how local signaling is achieved in the vertebrate retina.

# **Chapter 1**

## **Introduction**

The vertebrate retina is a part of the brain that detects patterns of photons in the environment and converts that signal into patterns of neural activity that can be interpreted by other visual centers in the brain. The vertebrate retina has a laminar structure with two synaptic layers, the outer and the inner plexiform layers (OPL and IPL respectively), intercalated between three cellular layers. The three cellular layers are the outer and inner nuclear layers and the ganglion cell layer. The retina has six general classes of cells: photoreceptors, horizontal, bipolar, amacrine, interplexiform, and ganglion cells. Despite abundant information about the circuitry of the vertebrate retina (Wilson, 2003), the modulation and function of some of these cell types is still poorly understood. Amacrine cells are the most diverse group of cells within the retina with respect to morphology and probably function (MacNeil and Masland, 1998) and these cells are the focus of my research. Amacrine cells are interneurons that receive synapses from bipolar cells and other amacrine cells and make synapses onto bipolar, ganglion cells and other amacrine cells (Ehinger, 1983). The main objective of this research is to learn more about  $\text{Ca}^{2+}$ -dependent signaling in retinal amacrine cells. In chapter 2, I describe the subcellular distribution of a type of glutamate receptor that mediates  $\text{Ca}^{2+}$  signaling. In chapter 3, I explore the role of mitochondria in spatial and temporal aspects of glutamate-dependent  $\text{Ca}^{2+}$  signals as well as the way in which mitochondria and the endoplasmic reticulum (ER) interact.

## **IMPORTANCE OF METABOTROPIC GLUTAMATE RECEPTORS IN THE RETINA**

Glutamate receptor activity and subsequent  $\text{Ca}^{2+}$  signaling control some of the major activities that take place in the vertebrate retina (Muller et al., 1992). Previous studies at the light microscopic level from the Gleason lab indicate that Group I metabotropic g-protein coupled glutamate receptors are expressed by cultured GABAergic amacrine cells and other cell types in the chicken retina (Kreimborg et al., 2001). It has also been demonstrated that

activation of these receptors generates  $\text{Ca}^{2+}$  signals in cultured retinal amacrine cells (Sosa et al., 2002). My goal in this study is to examine the subcellular localization of Group I metabotropic glutamate receptors throughout the retina and more specifically, to determine whether these receptors are expressed at amacrine cell synapses.

Glutamate is released from photoreceptors onto bipolar cells and horizontal cells in the OPL, and from bipolar cells onto amacrine cells and ganglion cells in the IPL (Copenhagan, 2003). The two major types of glutamate receptors expressed in the retina are ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Amacrine cells are known to express iGluRs (NMDA, AMPA, Kainate) (Hartveit and Veruki, 1997; Maguire, 1999; Tran et al., 1999). These receptors include NMDA and non-NMDA receptors that directly gate cation channels on amacrine cells (Hartveit and Veruki, 1997; Qin and Pourcho, 1999a, 1999b, 2001; Li et al., 2002) and can produce excitation.

The effects of activating mGluRs are, however, potentially much more diverse. These receptors alter ion channel function and other cellular processes indirectly by activating second messenger pathways. Metabotropic glutamate receptors are g-protein-coupled receptors, associated with activation of phospholipases, adenylyl cyclases and other second messenger systems. Eight subtypes of mGluRs can be further classified into three groups based on their amino acid sequence and pharmacology (Conn and Pin, 1997). Metabotropic glutamate receptors share about 70% sequence similarity within these groups, whereas between groups, the similarity averages about 45% (Nakanishi, 1992). Group I consists of mGluRs 1 and 5, Group II consists of mGluRs 2 and 3, Group III consists of mGluRs 4, 6, 7 and 8. Previous studies have already established the expression of mGluRs in the mammalian retina (Nomura et al., 1994; Hartveit et al., 1995; Brandstätter et al., 1996; Koulen et al., 1997; Vardi and Morigiwa, 1997; Cai and Pourcho, 1999; Vardi et al., 2000; Dyka et al., 2004). Group I mGluRs (1 and 5) and

Group II mGluRs (2 and 3) have been localized at the synaptic layers of the rat and cat retina (Cai and Pourcho, 1999; Koulen et al., 1997). The study reported here addresses whether Group I receptors are localized at synaptic sites throughout the chicken retina and also specifically addresses whether they are present at amacrine cell synapses. This is important with respect to understanding the role of these receptors in retinal signal processing. Metabotropic glutamate receptors 1 and 5 have been shown to activate phospholipase C (PLC) through coupling with  $G_q$  protein (Abdul-Ghani et al., 1996). Other studies have indicated that Group I receptors may also be coupled to adenylyl cyclase (Aramori and Nakanishi, 1992). Group I mGluRs (mGluR1 and 5) are linked to activation of the 1, 3, 4-inositol trisphosphate ( $IP_3$ ) pathway and production of cytosolic  $Ca^{2+}$  elevations (Conn and Pin, 1997). It has been previously demonstrated that activation of mGluR 5 induces  $Ca^{2+}$  elevations in cultured chick GABAergic amacrine cells (Kreimborg et al., 2001; Sosa et al., 2002). These  $Ca^{2+}$  elevations are temporally variable from cell to cell. This temporal diversity may be related to different patterns of  $Ca^{2+}$  influx as well as different patterns of  $Ca^{2+}$  buffering.

## **ROLE OF MITOCHONDRIA IN CALCIUM SIGNALING IN RETINAL AMACRINE CELLS**

Chapter 3 addresses the role of mitochondrial  $Ca^{2+}$  transport in shaping  $Ca^{2+}$  signals in amacrine cells.  $Ca^{2+}$  serves as an intracellular second messenger and exerts its influence on cells through changes in its concentration. Neuronal signaling, including receptor-mediated amacrine cell signaling often involves elevation of cytosolic  $Ca^{2+}$ . Two major ways cytosolic  $Ca^{2+}$  can be increased in a cell are the release of  $Ca^{2+}$  from intracellular stores (mitochondria, ER and nuclear envelope), and entry of  $Ca^{2+}$  from outside. Several  $Ca^{2+}$  buffering mechanisms present in the cell regulate changes in cytosolic  $Ca^{2+}$  levels. These include cytosolic  $Ca^{2+}$  binding proteins, uptake

and release of  $\text{Ca}^{2+}$  from mitochondria and ER and export of  $\text{Ca}^{2+}$  across the plasma membrane via  $\text{Ca}^{2+}$  ATPase and a  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger (Verkhratsky and Peterson, 1998).

In order to understand the properties of signaling within and between amacrine cells, a simplified culture system has been employed to study GABAergic,  $\text{Ca}^{2+}$ -dependent synaptic transmission between pairs of identified amacrine cells and  $\text{Ca}^{2+}$  signaling in single amacrine cells. Previous work in the Gleason lab has demonstrated that mitochondrial  $\text{Ca}^{2+}$  transport influences  $\text{Ca}^{2+}$  elevations induced by depolarization (Medler and Gleason, 2002). The  $\text{Ca}^{2+}$  elevations produced by glutamate have also been examined (Fillette and Gleason, unpublished observation) and measurements at the cell body indicate that mitochondria also influence these elevations. Here I address the role of mitochondria in buffering  $\text{Ca}^{2+}$  loads induced by glutamate stimulation in amacrine cell bodies and in cell processes. I extended my study to determine the physiological relationship between mitochondria and ER in amacrine cells. Together, these results will help us to understand the dynamics of  $\text{Ca}^{2+}$  signaling in retinal amacrine cells.

Mitochondria are commonly referred to as “the powerhouse of the cell” because they play an important role in converting energy generated from oxidation reactions into ATP in eukaryotic cells. Mitochondria also take part in enforcing programmed cell death (apoptosis) through a series of events in the cell (Duchen, 2000). These organelles also play a vital role in  $\text{Ca}^{2+}$  signaling and can participate directly in cellular  $\text{Ca}^{2+}$  responses (Rizzuto et al., 2000). Mitochondrial  $\text{Ca}^{2+}$  management involves both  $\text{Ca}^{2+}$  influx and calcium efflux. The outer mitochondrial membrane is freely permeable to  $\text{Ca}^{2+}$  ions but the inner mitochondrial membrane contains machinery to control the movement of  $\text{Ca}^{2+}$  into, and out of, the mitochondrial matrix.  $\text{Ca}^{2+}$  influx occurs via the  $\text{Ca}^{2+}$  uniporter. The uniporter operates as a  $\text{Ca}^{2+}$  channel and the movement of  $\text{Ca}^{2+}$  depends on the electrochemical gradient for  $\text{Ca}^{2+}$  between the cytosol and the mitochondrial matrix. The electrochemical gradient is dependent upon the mitochondrial

membrane potential ( $\sim -180\text{mV}$ ) generated by the proton gradient across the inner mitochondrial membrane. Studies on neuronal cells indicate that this mechanism can influence both the amplitude and time course of  $\text{Ca}^{2+}$  elevations in the cytosol (Friel and Tsien, 1994; Thayer and Miller, 1990; Medler and Gleason, 2002). This uniporter is thought to play a key role in some (Alnaes and Rahamimoff, 1975; Molgo and Picot-Dechavassine, 1998; Medler and Gleason, 2001), but not at all synapses (Zenisek and Matthews, 2000). The uptake of  $\text{Ca}^{2+}$  through the uniporter can be blocked by application of protonophores, such as p-trifluoromethoxy-phenylhydrazone (FCCP) and m-chlorophenylhydrazone (CCCP) (Friel and Tsien, 1994; Babcock and Hille, 1998).

The two mechanisms that are involved in the efflux of  $\text{Ca}^{2+}$  in excitable cells are the mitochondrial Na-Ca exchanger (mNCX) and the permeability transition pore (PTP) (Duchen, 2000). With the help of the  $\text{Na}^+$  gradient between the cytosol and mitochondrial matrix, the mNCX moves  $\text{Ca}^{2+}$  back into the cytosol. One way to inhibit this efflux mechanism is by the use of CGP37157, a specific blocker of mNCX in heart cells (Cox et al., 1993) that also blocks mNCX in neurons (White and Reynolds, 1997). The second efflux mechanism is the PTP, a high conductance, non-selective channel. Under extreme circumstances it is activated by a very large  $\text{Ca}^{2+}$  flux into the mitochondrial matrix. This mechanism facilitates the process of necrosis and apoptosis (Duchen, 2000).

Not only do mitochondria influence cytosolic  $\text{Ca}^{2+}$  levels during stimulation, they also interact with  $\text{Ca}^{2+}$  transport mechanisms on the endoplasmic reticulum. On stimulation, the ryanodine receptors (RyRs) and  $\text{IP}_3$  receptors ( $\text{IP}_3\text{Rs}$ ) mediate  $\text{Ca}^{2+}$  release from ER. Previous studies performed on a variety of cell types have suggested that mitochondria interact physically as well as physiologically with ER (Simpson et al., 1997; Rizzuto et al., 1998). Physical association of ER and mitochondria has been reported in multiple cell types. Clusters of  $\text{IP}_3$



receptors are located next to mitochondria in Purkinje neurons (Otsu et al., 1990; Satoh et al., 1990) and in glial cells (Simpson et al., 1997). Ryanodine receptors have also been located near mitochondria in muscle cells (Szalai et al., 2000). Furthermore, mitochondrial  $\text{Ca}^{2+}$  transport also regulates the sensitivity of  $\text{IP}_3$  receptors to  $\text{IP}_3$  (Hajnóczky et al., 1999; Gilibert et al., 2001; Jouaville et al., 1995; Landolfi et al., 1998; Csordas et al., 1999). Recent studies reveal a close physiological relationship between mitochondria and ryanodine receptors contributing to  $\text{Ca}^{2+}$  signaling in multiple cell types (Hajnóczky et al., 2002, Pacher et al., 2000, Szalai et al., 2000, Csordas et al., 2001, Nassar and Simpson, 2000, Krizaj et al., 2003). Here we investigate how mitochondria interact with ER in amacrine cells, and how this interaction affects resting  $\text{Ca}^{2+}$  levels in amacrine cells.

## **LOCAL CALCIUM SIGNALING IN RETINAL AMACRINE CELLS**

Amacrine cells are diverse interneurons and are thought to play an important role in local  $\text{Ca}^{2+}$  signaling in the retina (Euler et al., 2002). The extreme anatomical diversity of amacrine cells has been studied in some detail and characterized at least twenty two types of amacrine cells in the rabbit retina have been characterized based on the area of spread and levels of stratification within the IPL (MacNeil and Masland, 1998). Evidence of local  $\text{Ca}^{2+}$  signaling have been reported where the spatial variations in cytosolic  $\text{Ca}^{2+}$  signals occurred over short distances of  $<10\text{ }\mu\text{m}$  in intact tiger salamander retina (Denk and Detwiler, 1999). Previous studies in the Gleason lab have demonstrated that mitochondrial  $\text{Ca}^{2+}$  uptake, inhibited by application of FCCP, a protonophore that disrupts the membrane potential across the inner mitochondrial membrane, results in cytosolic  $\text{Ca}^{2+}$  elevations (Medler and Gleason, 2002). These FCCP-dependent cytosolic  $\text{Ca}^{2+}$  elevations may be local. Measurements of fluorescence intensity over time from cell bodies and processes indicate that  $\text{Ca}^{2+}$  elevations in processes did not follow the elevations at the cell bodies but were initiated simultaneously in both the cell

bodies and cell processes. This indicates that diffusion of  $\text{Ca}^{2+}$  from the cell body was not the source of  $\text{Ca}^{2+}$  elevation in processes (Medler and Gleason, 2002). Furthermore, in some cells  $\text{Ca}^{2+}$  elevations were detected in the cell body but not in the process of the same cell. This may be due to the non-uniform distribution of mitochondria in the amacrine cells. Because mitochondria are spatially discrete and because they are capable of temporarily sequestering large loads of  $\text{Ca}^{2+}$  (Herrington et al., 1996), mitochondria are good candidates for regulating local  $\text{Ca}^{2+}$  levels. The objectives of this study are two-fold. First, the consequences of the non-uniform distribution of mitochondria in shaping both spatial and temporal  $\text{Ca}^{2+}$  responses in amacrine cell processes are addressed. Second, the physiological relationship between mitochondria and ER in amacrine cells is also examined. These studies indicate that  $\text{Ca}^{2+}$  levels can be influenced by the distribution of mitochondria and also emphasize that mitochondria can have local effects on cytosolic calcium in amacrine cell processes. Moreover, these studies indicate clearly that mitochondria and the ER have the potential to interact physiologically during  $\text{Ca}^{2+}$  signaling in retinal amacrine cells.

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## **Chapter 2**

### **Immunolocalization of Metabotropic Glutamate Receptors 1 and 5 in the Synaptic Layers of the Chicken Retina\***

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## INTRODUCTION

Glutamate receptors play a major role in visual information processing in the vertebrate retina. In the outer plexiform layer (OPL) of the retina, glutamate is released from photoreceptors onto bipolar and horizontal cells. In the inner plexiform layer (IPL), glutamate is released from bipolar cells onto amacrine cells and ganglion cells. Two classes of receptors mediate the effects of glutamate: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors include NMDA and non-NMDA receptors that directly gate cation channels and mediate fast synaptic transmission (Hollman & Heineman, 1994). Metabotropic glutamate receptors are G-protein-coupled receptors that are linked to multiple signaling pathways including the inositol trisphosphate (IP<sub>3</sub>) and cyclic AMP pathways. The eight subtypes of mGluRs are further subdivided into three groups based on their amino acid sequence (Conn & Pin, 1997). Group I includes mGluRs 1 and 5, group II includes mGluRs 2 and 3, and group III includes mGluRs 4, 6, 7 and 8. Group I mGluRs have been shown to activate phospholipase C (PLC) and lead to elevations in IP<sub>3</sub> and cytosolic Ca<sup>2+</sup>. These receptors may also be coupled to activation of adenylate cyclase (Aramori & Nakanishi, 1992, Francesconi & Duvoisin 1998).

The presence of mGluRs in the mammalian retina has been clearly established through *in situ* hybridization and immunocytochemical studies (Nomura et al., 1994; Hartveit et al., 1995; Brandstätter et al., 1996; Koulen et al., 1997; Vardi & Morigiwa, 1997; Cai & Pourcho, 1999; Vardi et al., 2000; Dyka et al., 2004). Immunocytochemical studies performed at both light and electron microscopic levels in the rat and cat retina demonstrated that group I mGluRs are expressed at both synaptic layers and localized in the amacrine cell processes postsynaptic to bipolar cell terminals in the IPL (Koulen et al., 1997 and Cai & Pourcho, 1999). In the mouse retina, mGluR 1b and 5a/b expression has been localized to the OPL, the IPL and ganglion cells



at the level of the light microscope (Dyka et al., 2004). Another study, also at the light level, was done in the chicken retina and showed that Group I mGluRs are expressed extensively in this species, including both plexiform layers (Kreimborg et al., 2001). Although present in these synaptic layers, it has not yet been determined whether the receptors are expressed at synaptic sites rather than at more distant locations. Physiological experiments demonstrated that activation of group I mGluRs expressed by cultured retinal amacrine cells leads to  $\text{Ca}^{2+}$  elevations (Kreimborg et al., 2001) that are dependent upon activation of phospholipase C (Sosa et al., 2002). Thus, at least in this class of cell, group I mGluRs are linked to activation of the  $\text{IP}_3$  pathway.

In the present study, our objectives are two-fold: first, to determine whether group I mGluRs are localized to synaptic sites in the chicken retina, and second, to determine whether these receptors were localized to amacrine cell processes postsynaptic to bipolar cells. This second objective is central to understanding the implications of our physiological experiments in cultured amacrine cells. To achieve these objectives, we employed pre-embedding immunocytochemistry combined with electron microscopy to examine the localization of mGluRs 1 and 5. Here we show that Group I mGluRs are expressed at synapses in both the OPL and the IPL, including the synapses between bipolar cells and amacrine cells. This study also reveals distinct differences between distribution of mGluRs 1 and 5 in the chicken retina and some mammalian retinæ.

## **MATERIALS AND METHODS**

### **Antibodies**

Polyclonal antibodies against mGluR1 and 5 were purchased from Upstate Biotechnology (Lake Placid, NY). These antibodies were generated in rabbit against C-terminal peptide fragments of rat mGluR1 and 5. The mGluR1 antibody, was generated from the peptide

sequence (KPNVTYASVILRDYKQSSSTL), a sequence found only in the mGluR1a splice variant (Pin et al.1992), while the mGluR5 antibody was generated from the peptide sequence (KSSPKYDTLIIRDYTNSSSSL), present in both mGluR5a and mGluR5b splice variants (Joly et al.1995). The utility of these antibodies for mGluR 1 and mGluR5 in chicken tissue was previously established using Western blot analysis (Kreimborg et al., 2001). The anti-SV2 monoclonal antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA).

### **Tissue Preparation for Electron Microscopy**

Retinas from adult White Leghorn chickens were used in this study. Chickens were sacrificed by cervical dislocation followed by decapitation, a method approved by the Louisiana State University Animal Care and Use Committee in accordance with NIH guidelines. All manipulations were performed on tissue from at least three different chickens. Eyes were enucleated and the anterior portion and vitreous were removed. Eyecups were treated using a modified version of the procedure described by Brandstätter et al. (1996). Unless otherwise indicated, all materials were obtained from Sigma (St. Louis, MO). Eyecups were fixed in 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1 M Dulbecco's phosphate buffered saline (PBS, pH 7.4) for 15 minutes. This was followed by fixation in 4% paraformaldehyde in 0.1 M PBS, for two hours at room temperature. The eyecups were cryo-protected in 10% and 20% sucrose solutions for one hour each at room temperature followed by 30% sucrose solution at 4°C overnight. To increase the penetration of the antibodies, eyecups were subjected to three freeze-thaw cycles. Each freeze was for ten seconds on liquid nitrogen to increase the antibody penetration. After rinsing in 0.14 M PBS (pH 7.4), the retinae were dissected from the eyecups. The central retina was embedded in 4% agarose and vibratome-sectioned at a thickness of between 40 and 60  $\mu\text{m}$ .

## **Pre-embedding Immunoelectron Microscopy**

Vibratome sections were incubated for two hours in 10% normal horse serum (v/v) in PBS then incubated in the primary antiserum (1:200) against mGluR1 and mGluR5 for 4 days at 4°C. The tissue was then incubated for two hours at room temperature in biotinylated secondary antibody (1:100, Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA). Sections were rinsed in PBS and incubated with the ABC reagent for two hours at room temperature. After rinsing, sections were incubated in 3, 3'-diaminobenzidine (DAB) (Vector laboratories, Burlingame, CA) for ten minutes and then reacted in DAB with hydrogen peroxide. The reaction was stopped by rinsing in water then 0.1M cacodylate buffer (pH 7.4, Electron Microscopy Sciences, Fort Washington, PA). Sections were post-fixed in 2.5% (v/v) glutaraldehyde in cacodylate buffer for two hours at 4°C then rinsed in cacodylate buffer overnight at 4°C. The DAB reaction product was silver-intensified (Liposits et al., 1986) and then treated with 0.05% (w/v) gold chloride. Sections were then treated with 2% (w/v) OsO<sub>4</sub> in cacodylate buffer for an hour. Samples were dehydrated in graded series of ethanol (30%-100%) and flat-embedded in medium grade LR white (Electron Microscopy Sciences, Fort Washington, PA). The embedded tissue was sectioned then stained with 2% uranyl acetate and Reynolds' lead citrate (Bozzola & Russell, 1992). Several control experiments were performed on these samples. In the first, sections received all treatments except incubation with primary antibodies. In a second set of controls, sections were incubated with pre-immune serum in place of the primary antibodies. Another control experiment was done using the antigen peptide for the mGluR1 antibody that was provided by the manufacturer. Primary antibody (mGluR1) was pre-incubated with the mGluR1 peptide at an antibody/peptide ratio of 1:4 at 4°C overnight. This antibody-peptide mix was then diluted (1:200) just prior to use. The mGluR5 immunizing peptide was not available to perform a similar control for the mGluR5 antibody. Vibratome

sections used for control experiments were cut from the same blocks of tissue as those receiving primary antibodies. Vibratome sections were placed in control and experimental groups randomly.

Sections were examined and photographed using a Jeol EM 100 CX-II transmission electron microscope. The synaptic layers were examined at low power to locate synaptic ribbons. Only regions containing presynaptic ribbons were examined at high magnification and analyzed for labeling. For the purposes of quantification (Tables 1 and 2), “membrane labeling” was defined as the appearance of at least 10 grains of reaction product located on the plasma membrane. Labeling within 1.0  $\mu\text{m}$  of the ribbon was considered synaptic. Much of the labeling we observed was well within 0.5  $\mu\text{m}$  of the ribbon. Negatives were scanned and standard adjustments to brightness and contrast were made using Adobe Photoshop (San Jose, CA).

### **Light Microscopy**

Retinal tissue was obtained by the methods indicated in the previous section. After dissection, pieces of retina were fixed in 4% paraformaldehyde at 4°C for 2 hours. Following rinses in PBS, the tissue was incubated in 30% sucrose solution in PBS overnight at 4°C. Retinae were then frozen on a slurry of dry ice and isopentane and embedded in O.C.T. compound (Sakura Finetek, Torrance, CA). Retinal tissue sections were cut (14- 17  $\mu\text{m}$ ) on an American Optical cryostat. Double- labeling experiments were performed with monoclonal SV2 antibody and mGluR 1 and 5. The sections were incubated in 5% normal goat serum (NGS) in dilution solution for 1 hour. Sections were then incubated with SV2 (1:100) and mGluR1 and 5 (1:500) for 1 hour at room temperature. The secondary antibody used for SV2 labeling was goat-anti mouse Alexa Fluor 488 (1:800). Goat-anti rabbit Cy3 (1:800) was used as a secondary antibody for mGluR 1 and 5 labeling. Sections were washed in PBS and cover-slipped in mounting medium (70% glycerol, 28% PBS, 2% n-propylgallate, w/v) then viewed on an

inverted confocal microscope (Leica TCS SP2, 40X oil objective). Images of double-labeled material were acquired by sequential scanning to avoid bleed through and the pinhole was adjusted for optimal resolution using Leica LCS 'Lite' viewer software (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Standard adjustments to brightness and contrast were made in Adobe Photoshop.

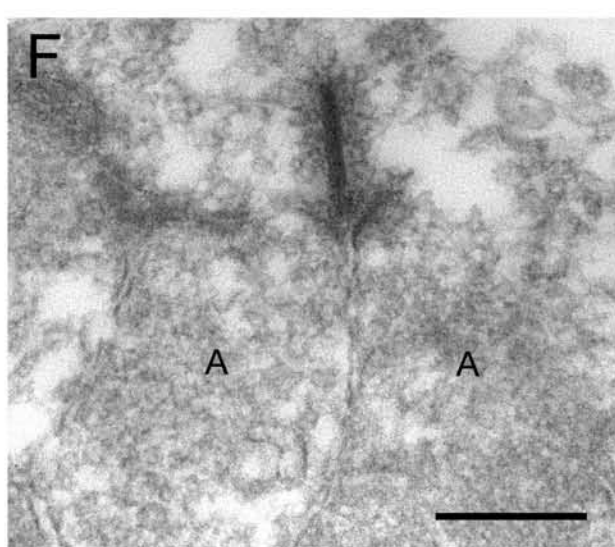
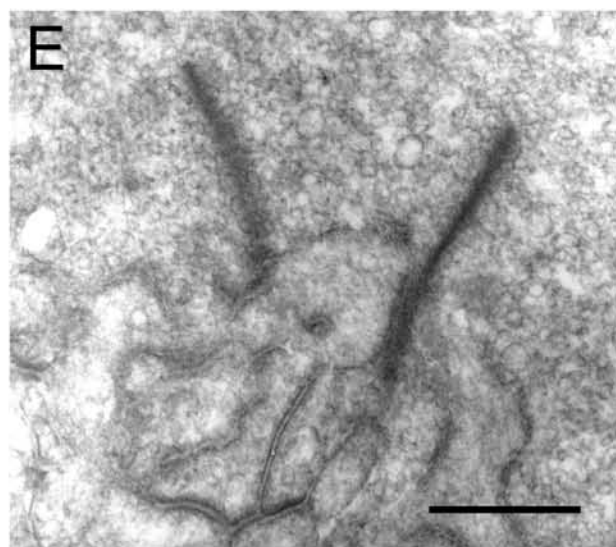
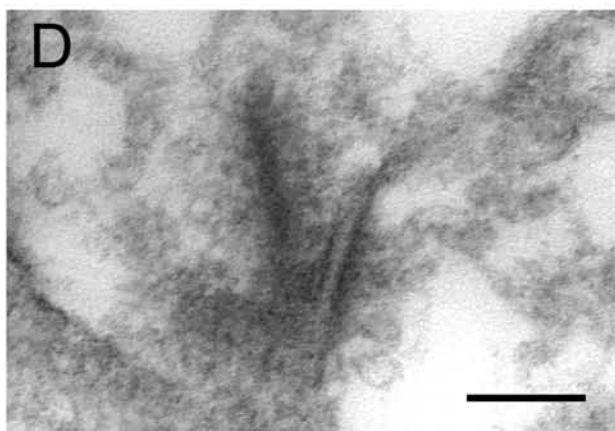
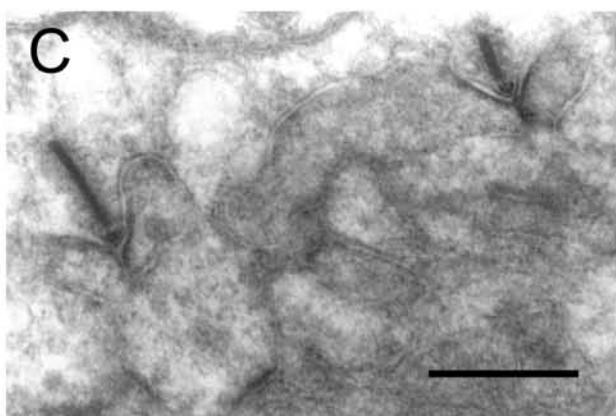
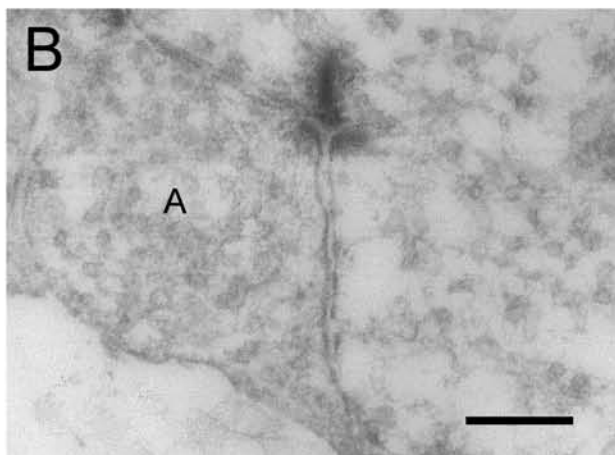
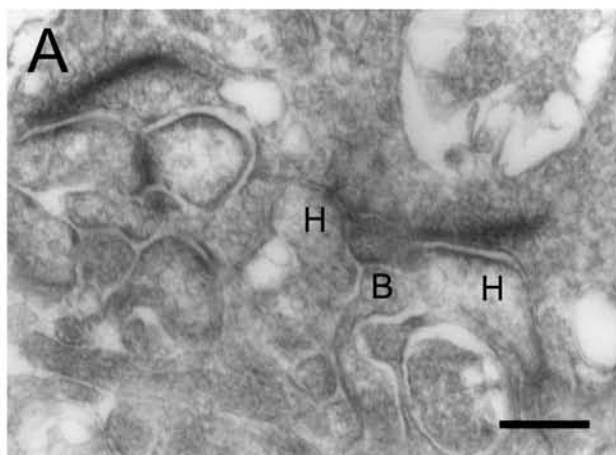
## **RESULTS**

All samples (including controls) were obtained from the central retina. Three types of control experiments were performed. In the first, all reagents were applied to the sections except for the primary antibodies (Fig 2.1A, B). In the second, sections were incubated with pre-immune rabbit serum instead of primary antibody (Fig. 2.1C, D). The third control involved pre-incubating the mGluR1 antibody with its peptide antigen (Fig 2.1E, F). No labeling was observed in any of these controls.

### **Localization of Metabotropic Glutamate Receptor 1**

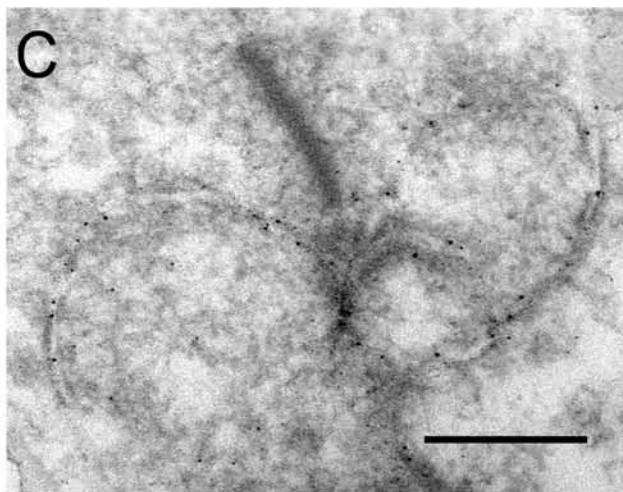
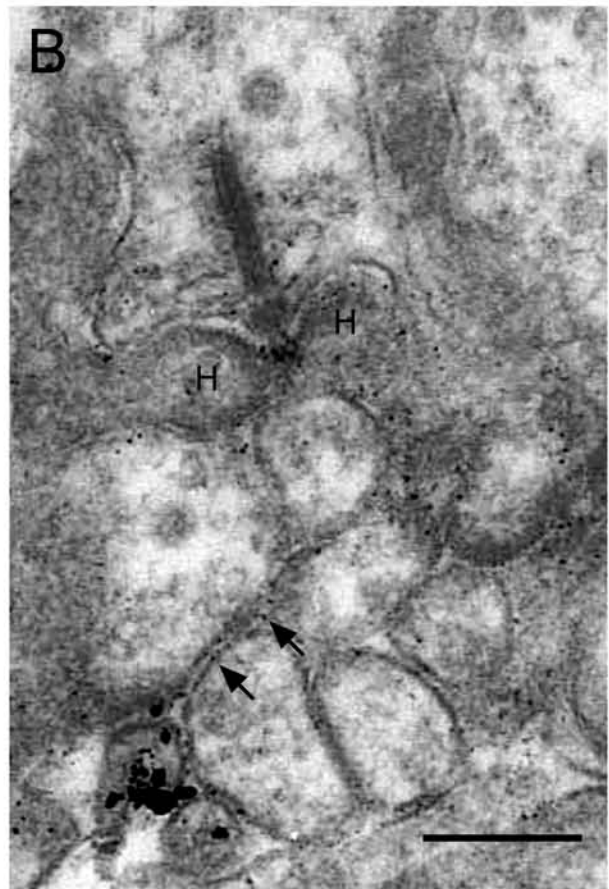
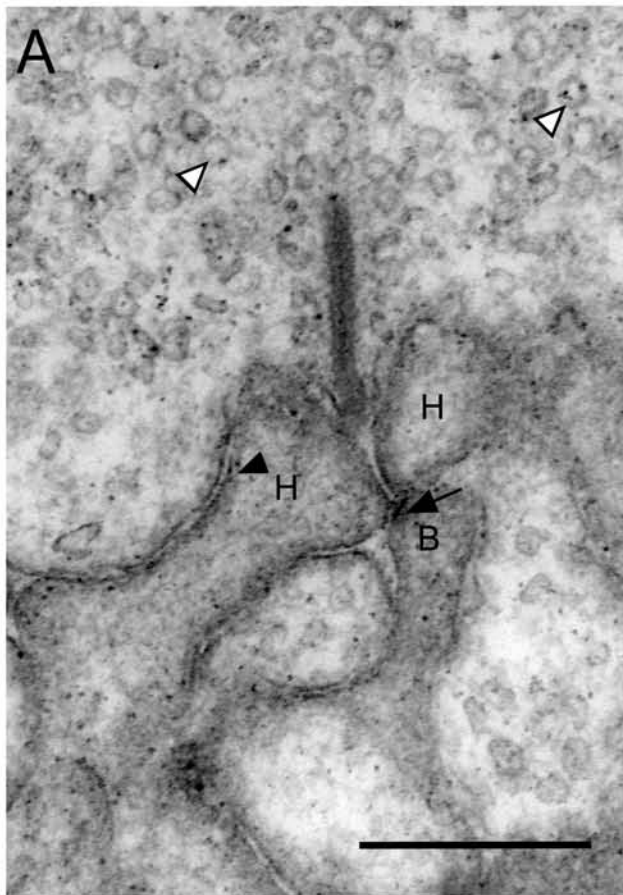
Anti-mGluR1 labeling was found presynaptically in some cone photoreceptors. Some label could be found on plasma membrane of cone pedicles near the ribbon (Fig. 2.2A, just above the filled arrowhead) but labeling found on vesicles in some cone pedicles was more convincing. Of the cone pedicles examined (n=62), 58% had labeling associated with presynaptic vesicles. All cone pedicles were associated with labeled postsynaptic elements (Table 2.1). Horizontal cell processes postsynaptic to cone photoreceptors expressed mGluR1 at the membrane (Fig. 2.2A (arrowhead), C). Metabotropic glutamate receptor 1 labeling was commonly most concentrated in between the two horizontal cells (Fig. 2.2B, C; 2.3A). Labeling was also found at the membranes of bipolar cell dendrites (Fig 2.2A, B). Although rods are much less prevalent than cones in the chicken retina, we occasionally observed photoreceptor

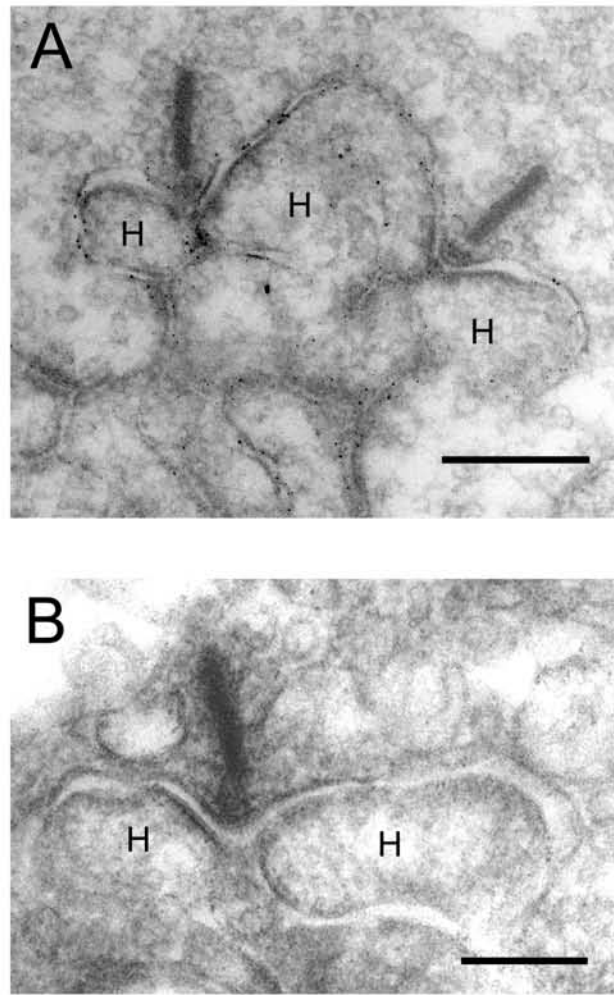
**Figure 2.1 Controls.** **A and B**, Control sections receiving all treatments except incubation with primary antibodies. **A**, In the outer plexiform layer (OPL) the horizontal cell processes (H) flank the ribbon in a cone pedicle and a bipolar cell dendrite (B) lies below the synaptic ribbon. **B**, In the inner plexiform layer (IPL) the ribbon synapse of a bipolar cell terminal is presynaptic to an amacrine cell process (A) as indicated by the presence of vesicles. No DAB reaction product is seen in either the OPL or IPL. **C and D**, Sections are incubated with pre-immune rabbit serum. **C**: Multiple ribbon synapses in the cone pedicle of the OPL shows no labeling. **D**, The ribbon synapse in the IPL is also free of the DAB reaction product. **E and F**, Sections treated with anti-mGluR1 preincubated with the antigen peptide. **E**, Multiple ribbon synapses in the OPL show no labeling. **F**, The ribbon synapse of a bipolar cell terminal presynaptic to amacrine cell processes is also unlabeled. Scale bars are 0.2  $\mu\text{m}$  except for **D** where the scale bar is 0.1  $\mu\text{m}$ .



**Figure 2.2 Localization of mGluR1 immunoreactivity in the OPL.** **A**, mGluR1 labeling is observed at the membrane of horizontal cell (arrowhead). Labeling is also observed on a bipolar cell dendrite (arrow). Some of the labeling is associated with the vesicles in the cone pedicle (open arrowheads). **B**, Labeling is also observed between two horizontal cell processes, at the membranes of bipolar cell dendrites (arrows) and inside a bipolar cell process (bottom, left). **C**, Another example of mGluR1 labeling of horizontal cell processes in the OPL. Scale bars are 0.2  $\mu\text{m}$ .







**Figure 2.3 Localization of mGluR1 immunoreactivity in the cone pedicles but not rod terminals.** **A**, mGluR1 labeling is observed at the membrane of horizontal cell processes postsynaptic to a cone pedicle containing multiple ribbons. **B**, No mGluR1 labeling is seen at photoreceptor synapses containing single ribbons, presumably rod terminals. Scale bars are 0.2  $\mu\text{m}$ .

terminals, presumably rods, which contained only single synaptic ribbons. Both pre- and postsynaptic labeling was always (Table 2.1) absent from these synapses (Fig. 2.3B).

**Table 2.1. mGluR1 and mGluR5 expression in the OPL of the chicken retina**

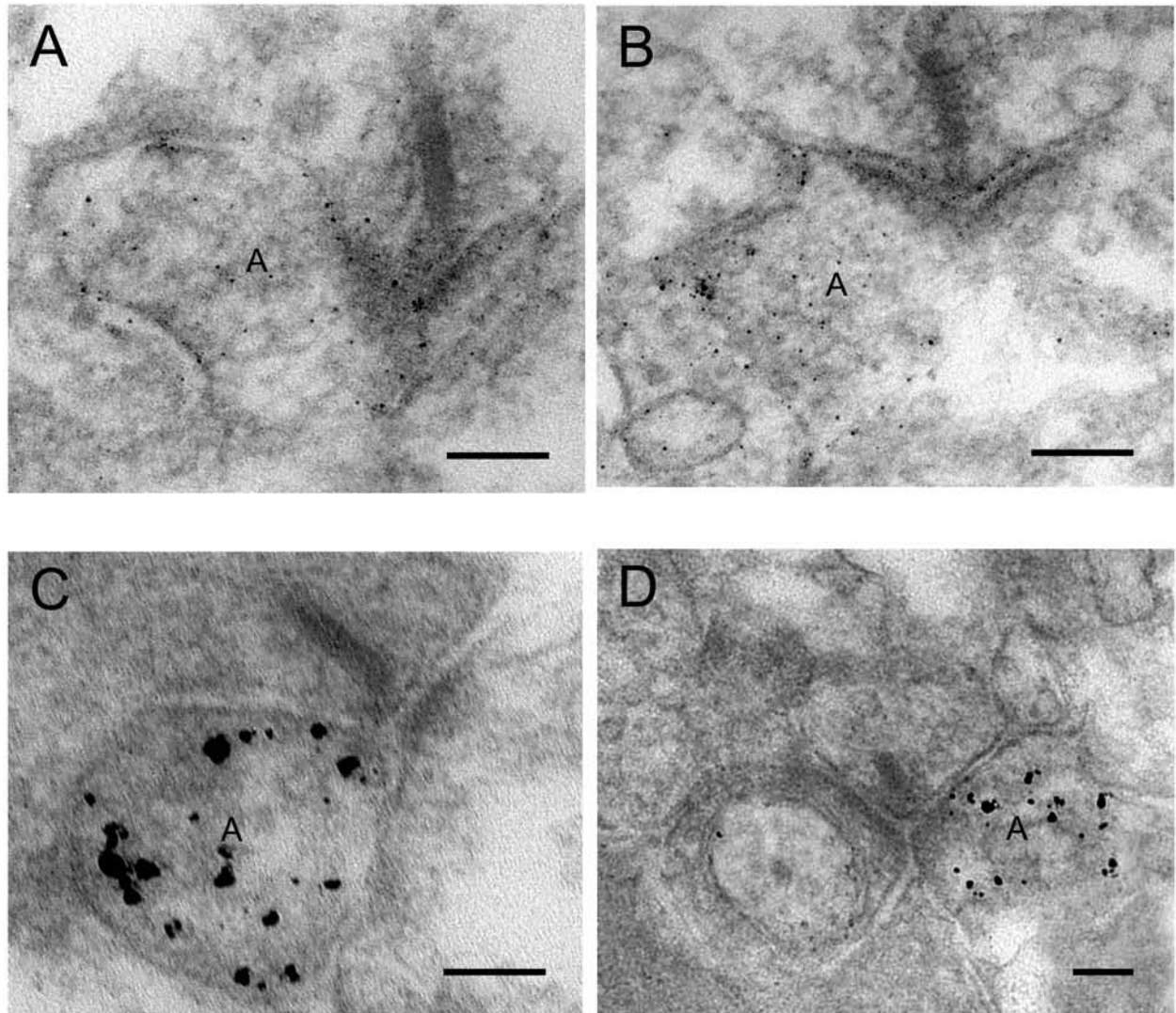
	<b>mGluR1</b> (n=62)	<b>mGluR5</b> (n=51)
% cone terminals with labeled vesicles	58	49
% cone ribbons with postsynaptic membrane labeling	100	100
% rod synapses labeled (n=15 each)	0	0

**Table 2.2. mGluR1 and mGluR5 expression in the IPL of the chicken retina**

	<b>mGluR1</b> (n=71)			<b>mGluR5</b> (n=58)		
% ribbons with presynaptic membrane labeling	18			17		
	Both	One	ND*	Both	One	ND*
% ribbons with postsynaptic membrane labeling	42	25	33	60	29	11
% ribbons with postsynaptic internal labeling	24	68	8	33	63	4

\* ND were denoted for synapses that could not be assigned to either category.

The unlabeled rod synapses were found in the same sections that contained labeled cone synapses. In the IPL, anti-mGluR1 labeling was predominately postsynaptic. However, labeling was clearly associated with presynaptic membranes in 18% of IPL synapses examined (Fig. 2.4B). Label clearly associated with presynaptic vesicle membranes was not observed at bipolar terminals. The pattern of postsynaptic labeling always fell into one of two distinct categories. In the first, labeling was found on the membranes of postsynaptic processes in combination with some scattered label inside the process (Fig. 2.4A, B). At other bipolar synapses, labeling was not found at the postsynaptic membrane but was instead concentrated inside one (Fig. 2.4C, D), or both elements of the dyad. When the labeling was localized to the postsynaptic membranes, it



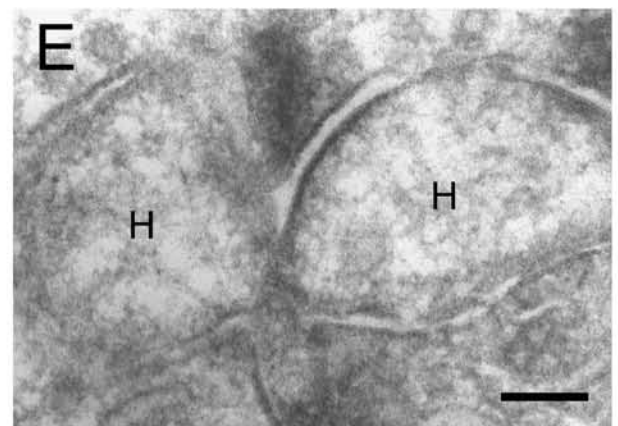
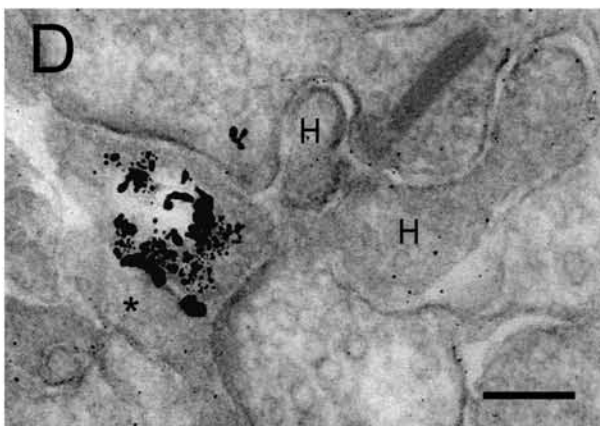
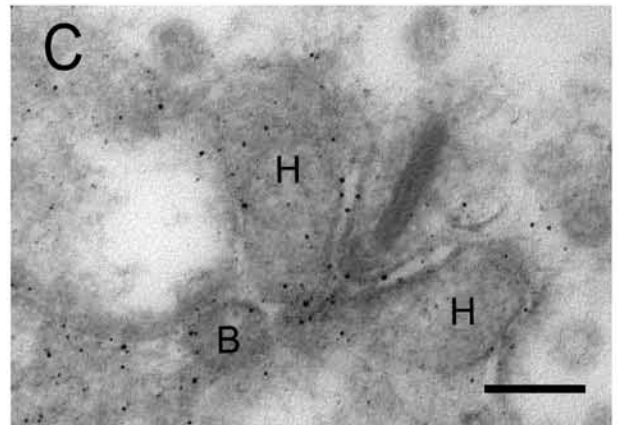
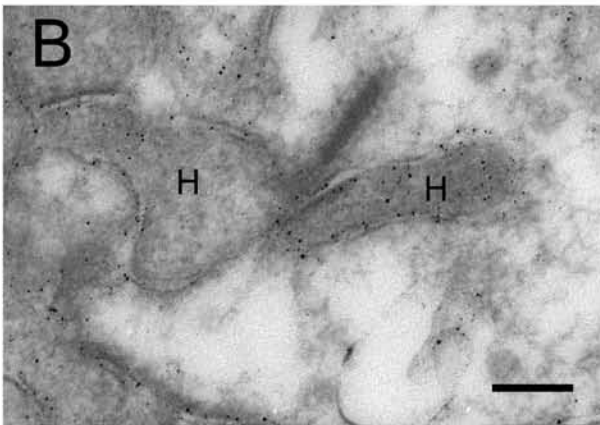
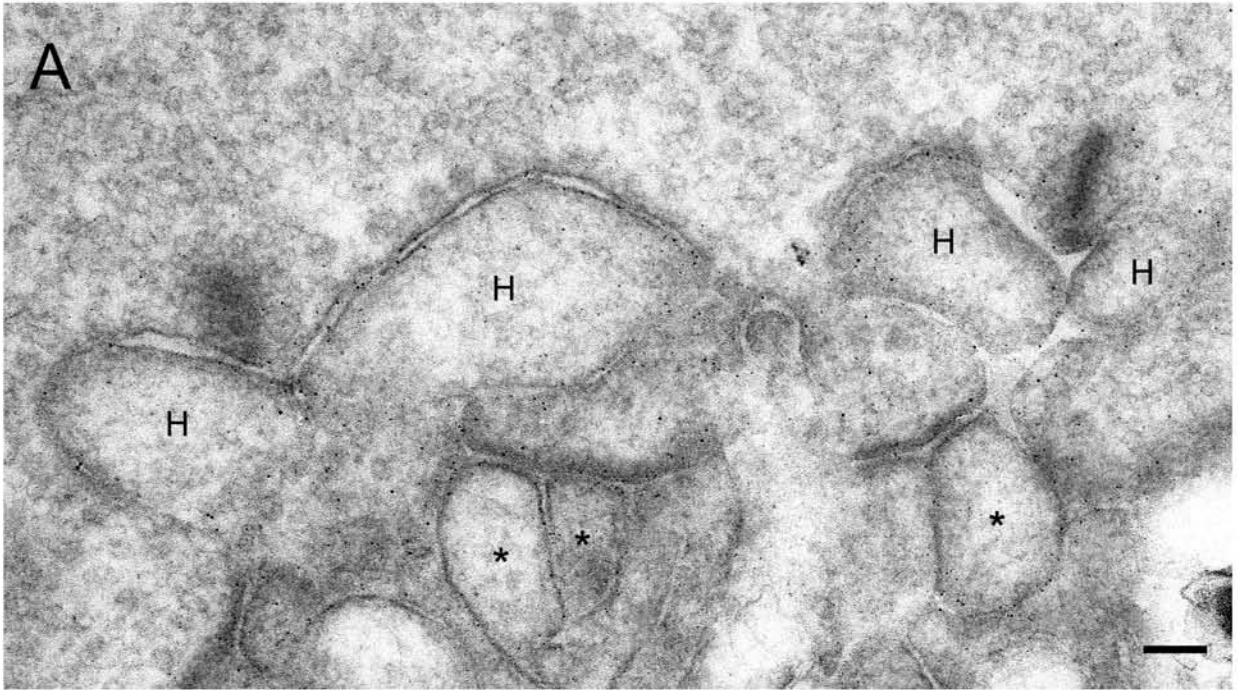
**Figure 2.4 Localization of mGluR1 immunoreactivity in the IPL.** **A**, Anti-mGluR1 labeling is seen at the membrane and inside an amacrine cell process postsynaptic to a bipolar cell. **B**, Anti-mGluR1 labeling is present at pre- and postsynaptic membranes near the ribbon synapse and also inside the amacrine cell process postsynaptic to the bipolar cell. **C**, Anti-mGluR1 labeling is concentrated inside an amacrine cell process. **D**, Anti-mGluR1 labeling is observed inside one element of the dyad (the amacrine cell), postsynaptic to the bipolar cell. Scale bars are 0.1  $\mu\text{m}$ .

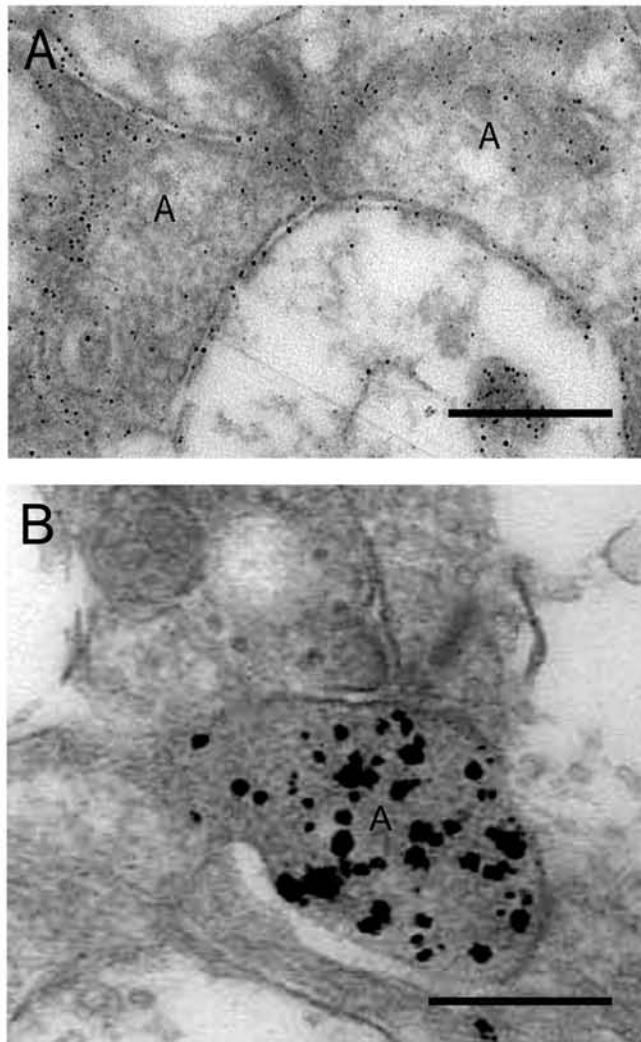
was most commonly located on both members of the dyad rather than just one (42% both vs 25% one, Table 2.2). For synapses with concentrated internal postsynaptic labeling, labeling at just Fig. 2.4 one member of the dyad was the more common observation (68% one vs. 24% both, Table 2.2). These processes consistently contained vesicles indicating that they are amacrine cells.

### **Localization of Metabotropic Glutamate Receptor 5**

Presynaptic vesicle labeling at cone pedicles was found in a similar fraction (49%) of cones with anti-mGluR5 as compared to anti-mGluR1 (58%) but the level of label within pedicles was lower with mGluR5 (Fig. 2.5A). Labeling could also be observed at the presynaptic membrane of cone pedicles (Fig. 2.5C). Metabotropic glutamate receptor 5 labeling was found postsynaptically at all cone synapses examined (n=51, Table 2.1) but at none of the rod synapses (Fig. 2.5E, n=15). As for anti-mGluR1, unlabeled rod synapses were found in the same sections containing labeled cone pedicles. At cone synapses, anti-mGluR5 labeling was observed at the membrane and inside horizontal cell processes (Fig. 2.5A-D). Bipolar cell processes (Fig. 2.5A, C) were also labeled with the anti-mGluR5 antibody. The concentrated mGluR1 labeling found between apposed horizontal cell processes was not observed with the mGluR5 antibody. Processes situated below the horizontal cell processes are most likely to be bipolar cell dendrites and labeling was also observed at these processes (Fig. 2.5A, D, asterisks). As for mGluR1, presynaptic mGluR5 labeling at bipolar cell terminals was relatively rare (Table 2.2). Anti-mGluR5 labeling was consistently found on processes postsynaptic to bipolar cell terminals. The postsynaptic labeling pattern was similar to that found for mGluR1 in that postsynaptic processes were either labeled at the postsynaptic membrane with some scattered interior labeling (Fig. 2.6A) or the processes contained a dense precipitate internally (Fig 2.6B). When the first pattern of labeling was observed, it was usually seen at both postsynaptic

**Figure 2.5 Localization of mGluR5 immunoreactivity in the OPL.** **A**, At a cone pedicle, mGluR5 labeling is observed at the membrane of the horizontal cell processes and other, probably bipolar cell, processes situated below the horizontal cell processes (asterisks). **B**, Anti-mGluR5 immunolabeling is seen at the horizontal cell membrane as well as inside the processes. **C**, Anti-mGluR5 labeling is seen at the membrane of horizontal cells and on a bipolar cell dendrite. **D**, Labeling is observed on horizontal cell processes and is also located internally in another process. **E**, No mGluR5 immunoreactivity is seen at synapses containing single ribbons. Scale bars are 0.1  $\mu\text{m}$ .





**Figure 2.6 Localization of mGluR5 immunoreactivity in the IPL.** **A**, A bipolar cell makes a ribbon synapse with two amacrine cell processes. Both members of this dyad are labeled with the antibody. **B**, Anti-mGluR5 labeling is observed inside an amacrine cell process postsynaptic to a bipolar cell terminal. Scale bars are 0.2  $\mu\text{m}$ .



processes of the dyad (60% both vs. 29% one) whereas the second pattern was more commonly found in only one member of a dyad (63% one vs. 33% both). As for mGluR1, the processes containing dense interior labeling could always be identified as amacrine cell processes. For both antibodies, the two patterns of labeling could be found in the same material indicating that these different patterns were not due to differences in antibody preparation or procedures.

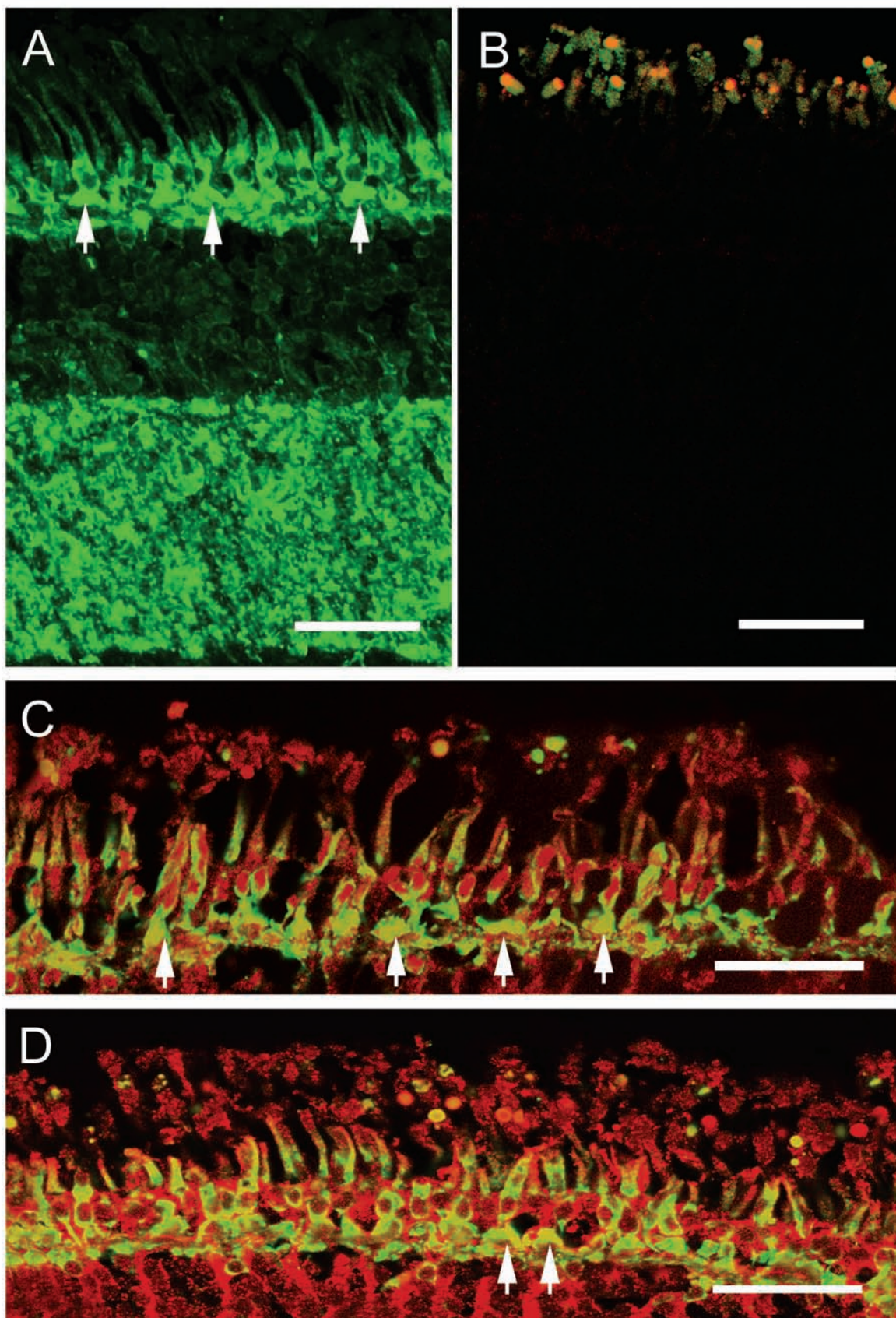
### **Localization of mGluR1 and mGluR5 to Cone Terminals**

To further examine the expression of group 1 mGluRs at cone terminals, we examined retinal sections at the light level. Our objective was to use a presynaptic marker to compare with the labeling pattern of mGluRs1 and 5. We performed double-labeling experiments with a monoclonal antibody raised against the transmembrane synaptic vesicle protein SV2 (Buckley & Kelly, 1985). Control experiments performed by omitting the primary antibodies gave no antibody labeling (Fig. 2.7B). In single labeling experiments, SV2 expression was observed in the inner region of photoreceptors including their terminals (Fig.2.7A, arrows) and throughout in the IPL. When double-labeled with mGluR1 and SV2, we observed co-localization of SV2 and mGluR1 labeling in the presynaptic terminals (Fig.2.7C, arrows). Similarly, mGluR5 and SV2 were observed to be co-localized in the photoreceptor terminals in the OPL (Fig.2.7D, arrows).

## **DISCUSSION**

We provide evidence that mGluRs 1 and 5 are expressed pre- and postsynaptically in the OPL and predominately postsynaptically in the IPL. Furthermore, the postsynaptic mGluRs can be localized to sites opposite, and immediately adjacent to, presynaptic sites suggesting that activation of the group I mGluRs can coincide with ionotropic glutamate receptor activation at these synapses. In this study we compared our results with results obtained at the light microscope level and also with the results obtained in the mammalian retinae.

**Figure 2.7 SV2 expression overlaps with mGluRs 1 and 5 in cone pedicles.** **A**, A confocal image showing the SV2 labeling pattern in the retina. Intense labeling is seen in the photoreceptors and photoreceptor terminals (arrows) as well as in the IPL. **B**, Confocal image of a control section that received both secondary antibodies incubation but no primary antibodies. No signal is seen in this section except auto-fluorescence from the photoreceptor oil droplets and outer segments. **C**, Anti-SV2 labeling (green) co-localizes with the anti-mGluR1 (red) labeling at the photoreceptor terminals (arrows, yellow). **D**, In a different section, anti-mGluR5 (red) labeling is shown to overlap with the SV2 labeling (green) at photoreceptor terminals (arrows). Scale bars are 50  $\mu\text{m}$ .



## **Comparison to Results at the Light Microscope Level**

The labeling pattern we reported previously at the light microscope level (Kreimborg et al., 2001) is, with one exception, in agreement with the pattern we report here. Some notable consistencies include the expression of mGluRs 1 and 5 in cone cells and in both bipolar cell dendrites and horizontal cell processes. In our previous work we observed labeling with both antibodies that was consistent with cone cell labeling. Here our results support and extend that observation with cone terminal labeling at the electron microscopic level and the overlap with SV2 expression at the light level. The occasional observation of horizontal cell bodies with anti-mGluR5-labeled processes previously observed at the light level gives additional support to our observation of horizontal cell process labeling by mGluR5 at the electron microscopic level. Expression of mGluRs 1 and 5 in bipolar cell dendrites is consistent with our previous work and confirmed by our observations in the electron microscope. Inconsistent with our previous work is our observation that mGluR1 and mGluR5 immunoreactivity was found primarily in the inner two-thirds of the IPL. In the light microscope, both antibodies produced labeling of discrete horizontal bands that spanned the entire width of the IPL. The most likely explanation for this discrepancy is the failure of our antibodies to penetrate to the center of the tissue. Unfortunately, attempts to further disrupt the tissue and increase antibody access resulted in an unacceptable loss of structure. Thus, we were unable to evaluate the distribution of these receptors in this subset of the IPL.

## **Comparison of the Localization of Group I mGluRs in Chicken and Mammalian Retinae**

When compared to the reports of similar experiments in cat (Cai & Pourcho, 1999) and rat (Koulen et al., 1997) retina, both similarities and differences in expression pattern are revealed. It should be noted that the mGluR1 antibodies used in all three studies were raised against a similar peptide fragment that is only found in the mGluR1 $\alpha$  splice variant for the

receptor. In the chicken retina, labeling for mGluR1 was consistently observed in cone pedicles but not in rod spherules. In the cat retina, mGluR1 immunoreactivity was found in rod spherules but not cone pedicles. No presynaptic labeling was reported in the rat OPL. In the chicken retina, both bipolar cell dendrites and horizontal cell processes were labeled. There is also immuno-cytochemical (Gafka et al., 1999) and physiological evidence (Linn & Gafka, 1999) for group I (mGluR1 $\alpha$  and mGluR5) expression by horizontal cells dissociated from the catfish retina. Postsynaptic OPL labeling was completely absent in the cat retina (Cai and Pourcho, 1999) and was confined to bipolar cells in the rat retina (Koulen et al., 1997). In the cat and rat retinæ, IPL labeling was confined to a single element of dyads at bipolar cell synapses, either an amacrine or ganglion cell process. The labeling pattern in the chicken IPL indicates that mGluR1 can be expressed at either one element of a dyad, always an amacrine cell process, or both.

The expression pattern of mGluR5 has been described for the rat retina (Koulen et al., 1997). The antibodies used in that study, however, were specific for the mGluR5a splice variant, whereas the antibodies used in this study were raised against a sequence found in both mGluRs 5a and b. This difference in antibody specificity allows for the possibility that elements in the rat retina negative for mGluR5a might express mGluR5b. In the OPL of the chicken retina, we find labeling at cone pedicles as well as in bipolar cell dendrites and horizontal cell processes. In the rat, however, OPL labeling is confined to bipolar cell dendrites. In the rat IPL, mGluR5 labeling is confined to single elements of dyads, whereas in the chicken IPL, labeling can be found at both members of a dyad.

Thus far, relatively few species have been examined for the expression of group I mGluRs. Within these species, however, considerable diversity is apparent. It is tempting to infer that the more widespread expression pattern found in the chicken retina relates somehow to the more complex retinal structure (Dowling, 1968; Dubin, 1970) and signal processing (Barlow,

1953; Maturana & Frenk 1963; Holden, 1977; Pearlman & Hughes, 1976; Hodos et al., 1982) found in non-mammalian retinas. Elucidation of expression patterns of these receptors in retinas of other non-mammalian species will be informative in this regard.

### **Presynaptic Labeling of Cone Pedicles**

Labeling was found in, and sometimes at the membrane of, cone pedicles for both mGluRs 1 and 5. The intensity of this labeling, however, was strongest for mGluR1. The labeling of vesicles in cone terminals may indicate that presynaptic group 1 mGluRs participate in constitutive or agonist-stimulated endocytosis. Metabotropic glutamate receptor 1 has been shown to be internalized by an agonist-dependent mechanism (Dale et al., 2001; Iacovelli et al., 2003; Mundell et al., 2004) as well an agonist-independent pathway (Dale et al., 2001; Bhattacharya et al., 2004). In contrast, mGluR5 has only been demonstrated to be internalized by a constitutive, agonist-independent pathway (Fourgeaud et al., 2003; Bhattacharya et al., 2004). This difference in internalization pathways for mGluR1 and mGluR5 may contribute to our observation that vesicle associated labeling is more intense in mGluR1 labeled cone pedicles.

### **The Functional Significance of Group I mGluR Expression**

Although mGluR6 is well-established to mediate the ON bipolar cell responses (Nakajima et al., 1993), the exact function of other mGluRs expressed in the retina is less well-understood. A few themes, however, are emerging. There is good evidence that group III mGluRs act as auto-receptors on photoreceptor (Koulen et al., 1999; Hirasawa et al., 2002) and bipolar cell terminals (Awatramani & Slaughter, 2001; Higgs et al., 2002) to limit neurotransmitter release. Activation of group II receptors on photoreceptors can apparently have a similar effect (Higgs & Lukasiewicz, 2002). Group III receptors are also likely to mediate the modulation of an inward rectifier current in horizontal cells (Dixon & Copenhagen, 1997). In the inner retina, there is also evidence that groups II (Robbins et al., 2003) and III (Shen &

Slaughter, 1998) mGluRs modulate the function of voltage-gated  $\text{Ca}^{2+}$  channels in ganglion cells. Less is known about the function of group I receptors. There is evidence that activation of group I receptors enhances voltage-gated  $\text{Ca}^{2+}$  channel currents in dissociated horizontal cells (Linn & Gafka, 1999), an effect that is thought to alter the response properties of these cells. In wide-field amacrine cells, mGluR1-mediated  $\text{Ca}^{2+}$  elevations have been shown to inhibit GABA-gated currents (Vigh & Lasater, 2003). Activation of group I receptors has also been shown to suppress  $\text{Ca}^{2+}$  currents in dissociated ganglion cells (Akopian & Witkovsky, 1996).

The effects of activating these receptors have also been investigated on cultured GABAergic amacrine cells. In  $\text{Ca}^{2+}$  imaging experiments both constitutive and stimulated mGluR1 activity was shown to inhibit signaling via mGluR5 (Kreimborg et al., 2001). The prevalence of both receptor types at synapses in singly-labeled tissue suggests that mGluRs 1 and 5 are often co-expressed. It may be that in amacrine cells (and possibly other co-expressing cell types), receptor interactions suppress mGluR5-dependent effects, except under specific circumstances.

We have identified two targets of mGluR5-dependent signaling in cultured GABAergic amacrine cells. Selective activation of these receptors leads to the enhancement of the currents through  $\text{GABA}_A$  receptors (Hoffpauir & Gleason, 2002) and through L-type  $\text{Ca}^{2+}$  channels (Sosa & Gleason, 2004). Examining the modulation of amacrine cell  $\text{GABA}_A$  receptors under conditions where both mGluRs 1 and 5 are activated will help to define the circumstances under which group I mGluR signaling is activated in the inner retina.

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## **Chapter 3**

### **Local Consequences of Mitochondrial Calcium Transport in Retinal Amacrine Cells**

## INTRODUCTION

Mitochondria serve multiple cellular functions including the generation of ATP, the mediation of events leading to cell death and the transport of  $\text{Ca}^{2+}$ . Each of these functions involves a complex and sometimes overlapping set of molecular players. Although a variety of  $\text{Ca}^{2+}$  transport mechanisms had been well documented for mitochondria, it was not until the 1990's that it was established that this transport occurred under physiological conditions. Furthermore, it has become clear that, at least in some cell types, mitochondrial  $\text{Ca}^{2+}$  transport has a significant impact on cytosolic  $\text{Ca}^{2+}$  signaling.

Mitochondria express multiple  $\text{Ca}^{2+}$  transport mechanisms. The two most relevant to normal neuronal function are the  $\text{Ca}^{2+}$  uniporter and the mitochondrial sodium calcium exchanger (mNCX) (Gunter and Gunter, 1994; Babcock and Hille, 1998). The  $\text{Ca}^{2+}$  uniporter is proposed to be a  $\text{Ca}^{2+}$  channel and the rapid movement of  $\text{Ca}^{2+}$  across the inner mitochondrial membrane is driven by the steep electrochemical gradient for  $\text{Ca}^{2+}$ . The major contributor to this gradient is the large membrane potential across the inner mitochondrial membrane which is established by the activities of the electron transport chain. Subsequent efflux of  $\text{Ca}^{2+}$  from mitochondria is achieved via the mNCX. The operation of this mechanism provides a relatively slow release of  $\text{Ca}^{2+}$  into the cytosol. Thus, mitochondria have the potential to both limit the amplitude and time course of cytosolic  $\text{Ca}^{2+}$  elevations by rapid transport of  $\text{Ca}^{2+}$  into the mitochondrial matrix via the uniporter and to prolong a  $\text{Ca}^{2+}$  signal by more slowly re-releasing  $\text{Ca}^{2+}$  into the cytosol via the mNCX.

In addition to their role in regulating cytosolic  $\text{Ca}^{2+}$ , evidence is mounting that mitochondria interact both physically and physiologically with the endoplasmic reticulum (Rizzuto et al., 1998, 2004). Physical proximity of the ER and mitochondria has been demonstrated in multiple cell types. Clusters of  $\text{IP}_3$  receptors are localized next to mitochondria

in Purkinje neurons (Otsu et al., 1990; Satoh et al., 1990) and glial cells (Simpson et al., 1997). Additionally, it has been shown that mitochondrial  $\text{Ca}^{2+}$  transport can regulate the sensitivity of  $\text{IP}_3$  receptors (Jouaville et al., 1995; Landolfi et al., 1998; Csordas et al., 1999; Hajnoczky et al., 1999; Gilibert et al., 2001). In muscle cells, mitochondria are located near ryanodine receptors and in cardiac myocytes, mast cells, neurons and vertebrate retina  $\text{Ca}^{2+}$  signals from ryanodine receptors are transmitted to mitochondria (Friel, 2004; Hajnoczky et al., 2002; Pacher et al., 2000; Szalai et al., 2000; Csordas et al., 2001; Nassar and Simpson, 2000; Krizaj et al., 2003).

In some cell types, it is becoming clear that the location of mitochondria has more global effects on the spatial pattern of cytosolic  $\text{Ca}^{2+}$  elevations (Tinel et al., 1999). The consequences of localization have been best worked out in pancreatic acinar cells. These polarized secretory cells have three distinct zones of mitochondria and, it has been demonstrated that a belt of perigranular mitochondria limit the spatial extent of the  $\text{Ca}^{2+}$  waves that trigger exocytosis (Tinel et al., 1999). In contrast, relatively little is known about how mitochondria shape the spatial aspects of  $\text{Ca}^{2+}$  signaling in neurons. Some neurons, vertebrate skeletal motor neurons for example, are highly polarized with respect to their synaptic inputs and outputs. These neurons receive synaptic input at their dendrites in the CNS and send synaptic output to peripherally located target tissues. Interneurons, however, can be substantially less polarized in that their synaptic inputs and outputs can occur at nearby locations. Under these circumstances, spatially discrete mechanisms affecting cytosolic  $\text{Ca}^{2+}$  may be especially relevant.

Amacrine cells are retinal interneurons that have both their synaptic inputs and outputs within the inner plexiform layer of the retina. Amacrine cells are anatomically diverse neurons (MacNeil and Masland, 1998) and seem likely to perform similarly diverse functions in retinal signal processing, many of which are presently unknown. Much of what is known about amacrine cells suggests that they participate in local signaling. Examination of synaptic circuitry

of the inner plexiform layer at the EM level indicates that amacrine cell synaptic inputs and outputs are sometimes separated by less than a micron (Dowling and Boycott, 1966). It has also been demonstrated both anatomically and physiologically that amacrine cell processes post synaptic to bipolar cells send inhibitory feedback synapses back to the presynaptic bipolar cell terminal (Hartveit, 1999, Shields and Lukasiewicz, 2003). The idea that local,  $\text{Ca}^{2+}$ -dependent synaptic signaling occurs in amacrine cells is also supported by the detection of spatial inhomogeneities in cytosolic  $\text{Ca}^{2+}$  signals over short distances ( $<10\mu\text{m}$ ) in amacrine cell processes in the intact retina (Denk and Detwiler, 1999). Because mitochondria are usually spatially discrete and are capable of transporting  $\text{Ca}^{2+}$ , it is possible that they play a role in regulating local  $\text{Ca}^{2+}$  levels in amacrine cell processes.

We have previously shown that mitochondria influence the time course of depolarization-dependent cytosolic  $\text{Ca}^{2+}$  elevations in cultured chick amacrine cell bodies, where mitochondria are abundant (Medler and Gleason, 2002). Here we extend these studies to processes where the distribution of mitochondria is much more diverse with some processes of the same cell having numerous mitochondria and other processes having relatively few (see results). Specifically, we address two questions related to the local effects of mitochondrial calcium transport. First, we ask how mitochondrial  $\text{Ca}^{2+}$  transport functionally interfaces with  $\text{Ca}^{2+}$  transport mechanisms on the endoplasmic reticulum in cell bodies and processes. Second, we address the role of mitochondria in shaping both spatial and temporal aspects of the  $\text{Ca}^{2+}$  responses to glutamate in amacrine processes. To achieve these objectives, we combine mitochondrial localization with cytosolic  $\text{Ca}^{2+}$  imaging in amacrine cells derived from the chick retina.

## **MATERIALS AND METHODS**

In this study,  $\text{Ca}^{2+}$  imaging and immunocytochemistry was performed on cultured preparation of identified amacrine cells.

## **Cell Culture**

Retinas from 8-day old White leghorn chicken embryos (*Gallus gallus domesticus*, Poultry Science Department, Louisiana State University) were dissociated and cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Rockville, MD, USA) with 5% fetal bovine serum (HyClone, Logan, UT, USA), 1000 U penicillin/ml, 100 µg streptomycin/ml and 1mM L-glutamine (Sigma). The cells were fed every other day with Neurobasal Medium (1X), 1-2mM Glutamine Supplement and 1% B-27 Supplements (GIBCO Laboratories, Rockville, MD, USA). Cells were plated onto 0.1mg/ml poly L-ornithine coated coverslips.

## **Immunocytochemistry**

Cultured retinal amacrine cells grown on glass coverslips were fixed at embryonic equivalent (E.E) day 15 for 30 minutes in 2% paraformaldehyde. Fixed material was blocked in 5% normal goat serum in dilution solution (1% BSA, 0.1% saponin in PBS) for 30 minutes before incubation with primary antibody for one hour at room temperature. Cells were then washed three times with PBS before application of fluorescently-labeled secondary antibody for one hour followed by another PBS wash cycle. For double labeling experiments, this process was repeated for the second primary antibody. Coverslips were mounted on microscope slides in mounting medium (70% glycerol, 28% PBS, 2% n-propyl gallate) before viewing.

Polyclonal antibodies to IP<sub>3</sub> receptor and ryanodine receptor 3 were obtained from Calbiochem (San Diego, CA) and Chemicon (Temecula, CA) and applied at dilutions of 1:200 and 1:500, respectively. The monoclonal antibody is raised against a mitochondria-specific protein. Monoclonal mitochondrial antibody 4C7/2E4 was developed by Dr. Ronald Thurston and Nancy Korn at Clemson University and was obtained from the Developmental Studies Hybridoma Bank which was developed under the auspices of the NICHD and is maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. The mitochondrial



antibody was applied as undiluted supernatant. Polyclonal antibodies were labeled with goat-anti-rabbit Cy3 (Chemicon, 1:800) and mitochondrial monoclonal antibody was labeled with goat-anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR; 1:300). Labeling was visualized with an Olympus IX70 microscope and images were captured with a Sensicam QE (Cooke Corporation, Romulus, MI) and IPLabs software (Scanalytics, Rockville, MD).

To verify mitochondrial antibody labeling, cultured retinal amacrine cells were loaded for 15 minutes with Mitotracker Red (100nM, Molecular Probes) before fixation and labeling with mitochondrial antibody.

### **Localization of Mitochondria**

The cells were loaded with 20 nM MitoTracker Red for 15 minutes at room temperature. It has been established that this low concentration of MitoTracker Red does not interfere with mitochondrial function (Buckman et al., 2001). To eliminate errors in data collection due to mitochondrial movement (Morris and Hollenbeck, 1995), an image of the cell was recorded at the beginning and at the end of each experiment. Only data collected from processes with stationary mitochondria were used in the analyses.

### **Calcium Imaging**

Cultured cells (E.E. 16-18) plated onto coverslips were loaded with 2  $\mu$ M Oregon Green 488 BAPTA-1 AM (Molecular Probes) in Hank's balanced salt solution for 1 hour at room temperature. The cells were subsequently loaded with 20 nM MitoTracker Red (Molecular Probes) for 15 minutes. At the end of the loading period the cells were placed in an open chamber and observed using an inverted Leica TCS SP2 spectral confocal microscope (63X oil objective) equipped with Ar, He/Ne (green) and He/Ne (red) lasers. The image and data acquisition were performed using Leica LCS software packages. Data were collected from the cell bodies and from primary cell processes. In cell processes, data were collected from regions

either immediately adjacent to mitochondria or more than 10  $\mu\text{m}$  away from any mitochondrion. Background fluorescence, measured from regions of the field devoid of cells and their processes was subtracted from all data. Data were plotted with the ORIGIN software package (Microcal, Northampton, MA, USA). Statistical analyses were performed using paired (where appropriate) t-test. Data in bar graphs are reported as means and standard deviation. Time course of the responses were measured as the duration at half-peak amplitude. Relative changes in amplitudes of the responses were measured within cells.

### **pH Imaging**

Cultured retinal neurons were loaded with 2 $\mu\text{M}$  SNARF-1 AM for 1 hour, washed thoroughly with Hank's balanced salt solution, and mounted on an opening recording chamber. Fluorescence intensity over time was measured at two bands of emission wavelengths: 570-600nm and 630-660nm, using a Leica confocal microscope and excitation with the 543 laser line. An increase in fluorescence at the former and a decrease in the latter set of wavelengths would be consistent with cytosolic acidification whereas an increase at 630-660nm and a decrease at 570-600nm would be consistent with cytosolic alkalinization. Cells were kept under constant perfusion with normal external solution between drug applications. Fluorescence intensity data were subsequently analyzed using Origin 7.5 software. Data were reported as the ratio of fluorescence intensity at 570-600nm to fluorescence intensity at 630-660nm.

### **Ratiometric Analysis**

The regional differences in resting  $\text{Ca}^{2+}$  levels in processes were measured by Fura-2 AM imaging. The cells were loaded with 10  $\mu\text{M}$  Fura 2-AM for an hour, then with 20nM MitoTracker Red for 15 minutes at room temperature. Cells grown on coverslips were placed on a slide and viewed with a Leica DM RXA2 upright microscope, using an oil-immersion objective (1.3NA, X100). UV excitation was provided by a short arc xenon lamp (Sutter

Instruments, Novato, CA). In these experiments, data were collected by switching between 340nm and 380nm wavelengths using the software Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO). Fluorescence measurements were obtained from regions of processes next to the mitochondria and from regions at least 10  $\mu\text{m}$  away from the mitochondria.

## **Solutions**

Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Solutions were applied into the open chamber by a gravity-driven bath perfusion system. The flow of the solution was maintained at 2-3 ml/min and solutions were switched manually. Experiments were performed using the following external solution (in mM): KCl, 5.3, NaCl, 135.0,  $\text{CaCl}_2$ , 3.0,  $\text{MgCl}_2$ , 0.41, Glucose, 5.6, and HEPES, 3.0, pH 7.4. The following reagents were used: 30  $\mu\text{M}$  glutamate, 1  $\mu\text{M}$  Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 5  $\mu\text{M}$  7-chloro-5-(2-chlorophenyl)-1, 5-dihydro-4, 1-benzothiazepine-2(3H)-one (CGP, EMD Biosciences, Inc., San Diego, CA), 1 $\mu\text{M}$  Thapsigargin, 30  $\mu\text{M}$  Cyclopiazonic acid (CPA, Tocris Bioscience, Ellisville, MO) and 20  $\mu\text{M}$  ryanodine. The cells were exposed to glutamate for 8 seconds. FCCP and CGP exposures were for 1 minute. Cells pre-treated with thapsigargin were exposed to the inhibitor for one hour. Experiments with CPA were performed by exposing the cells to CPA for 5 minutes prior to imaging followed by a 1 minute application of CPA and then 1 minute of FCCP and CPA. Ryanodine was applied for 1 minute on the cells followed by a combined application of ryanodine with FCCP for one minute.

## **RESULTS**

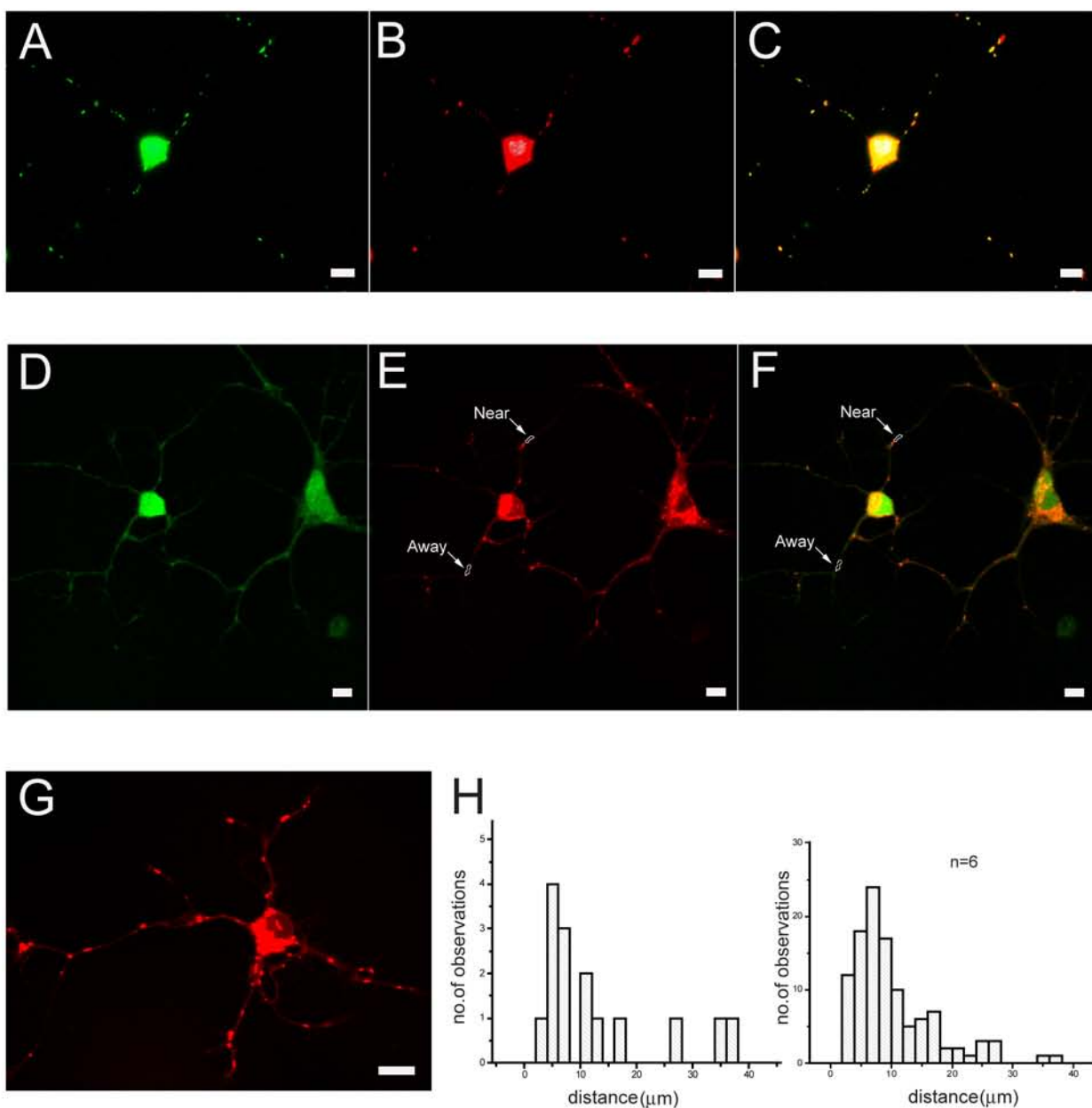
To confirm the utility of MitoTracker Red labeling as an indicator of mitochondrial position, the labeling pattern of MitoTracker Red was compared to the labeling pattern of a monoclonal antibody raised against mitochondria isolated from turkey spermatozoa (monoclonal

antibody 4C7/2E4). Mitochondria are abundant in the cell body but are typically unevenly distributed in cell processes. The overlay image of the MitoTracker Red dye and 4C7/2E4 (green) shows the extensive co-localization of the two types of label that we typically observed and verifies the utility of MitoTracker Red (Fig. 3.1C).

In order to evaluate the spatial impact of mitochondrial  $\text{Ca}^{2+}$  transport, cells were co-loaded with Oregon Green 488 BAPTA-1 AM (OGB 488) and MitoTracker Red. Amacrine cells are shown loaded with OGB 488 (Fig.3.1D), then with MitoTracker Red (Fig. 3.1E). Arrows (Fig. 3.1E, 3.1F) indicate typical regions of data collection from processes. The distributions of mitochondria in the processes were quantified to describe the typical mitochondrial distribution in amacrine cell processes (Fig 3.1 G, H).

### **FCCP-dependent $\text{Ca}^{2+}$ Elevations in Amacrine Cells**

We have previously shown that FCCP produces  $\text{Ca}^{2+}$  elevations that are independent of extracellular  $\text{Ca}^{2+}$  (Medler and Gleason 2002). Because FCCP is a protonophore, one potential difficulty with this approach is that disrupting the proton gradient across the inner mitochondrial membrane will allow the mitochondrial ATP synthase to consume ATP rather than synthesize it. Previous work, however, has established that with the brief durations (1 min) of FCCP application used in these experiments, ATP depletion does not affect the properties of cytosolic  $\text{Ca}^{2+}$  elevations in cultured amacrine cells (Medler and Gleason, 2002). These  $\text{Ca}^{2+}$  elevations were further characterized to determine whether they might reveal something about the function of mitochondrial  $\text{Ca}^{2+}$  transport in amacrine cells. Figure 3.2 shows typical FCCP-dependent  $\text{Ca}^{2+}$  produced in cell bodies (Fig. 3.2A) and processes (Fig. 3.2D). Second FCCP applications (1 min. after recovery from first FCCP application) on cell bodies (Fig. 3.2B) and processes (Fig. 3.2E) produced  $\text{Ca}^{2+}$  elevations that were similar in amplitude (cell bodies  $p=0.090$ ; cell processes  $p=0.181$ ) and not significantly different in duration (cell bodies  $p=0.831$ ; cell

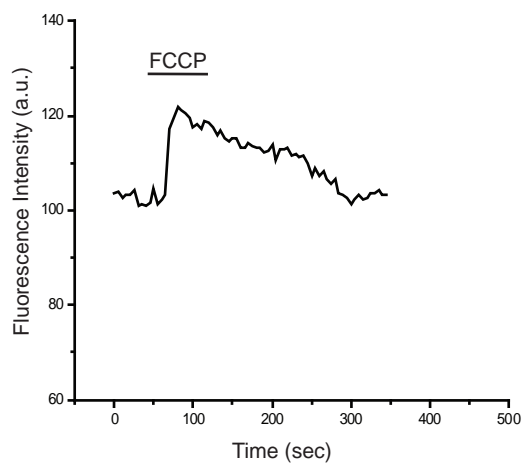


**Figure 3.1 Distribution of mitochondria in cultured amacrine cells.** **A**, An amacrine cell labeled with mitochondrial antibody 4C7/2E4. **B**, The same cell labeled with MitoTracker Red. **C**, An overlay image of mitochondrial antibody and mitochondrial dye in the amacrine cell showing co-localization. **D**, Amacrine cells are loaded with Oregon Green 488 BAPTA-1 AM. **E**, The cells are also loaded with MitoTracker Red. **F**, An overlay image of the amacrine cells co-loaded with both the indicators. Arrows (E and F) indicate typical regions of data collection. **G**, An example of mitochondrial distribution in amacrine cell processes. **H**, Frequency histograms depicting the distribution of mitochondria in a single cell (left) and collectively from 6 cells (right). The distributions of distances between mitochondria in processes are plotted. **A-C** contributed by Emily McMains. Scale bars are 10 μm.

**Figure 3.2. FCCP-dependent  $\text{Ca}^{2+}$  elevations in amacrine cells.** **A-C**, FCCP-dependent  $\text{Ca}^{2+}$  elevations measured in cell bodies. **A**, FCCP-dependent  $\text{Ca}^{2+}$  elevation is elicited in an amacrine cell body upon application of FCCP (1 minute). **B**, A second application of FCCP (delivered 1 minute after recovery) produces  $\text{Ca}^{2+}$  elevation similar to that of the first application. **C**, Relative amplitude ( $p=0.090$ ) and duration (measured at half-height) ( $p=0.831$ ) of these responses are not statistically different. **D-F**, FCCP-dependent  $\text{Ca}^{2+}$  elevations measured in processes. **D**, An FCCP-dependent  $\text{Ca}^{2+}$  elevation is measured in a region immediately adjacent to mitochondria (black trace) but little or no response is detected in a region  $> 10 \mu\text{m}$  away from a mitochondrion (gray trace). **E**, Second applications of FCCP produce  $\text{Ca}^{2+}$  responses in regions of processes adjacent to mitochondria. **F**, Relative amplitudes ( $p=0.181$ ) and durations ( $p=0.947$ ) of first and second responses measured in regions next to mitochondria are not statistically different. “a.u.” stands for arbitrary units.

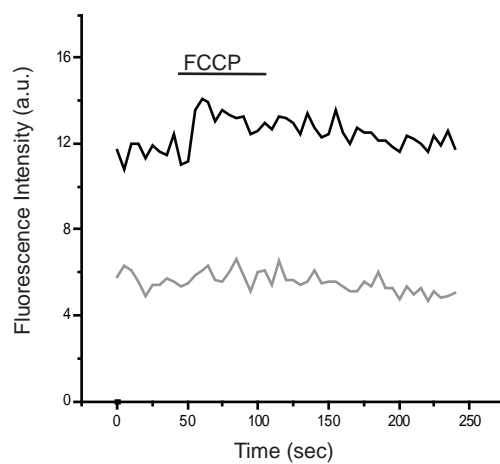
A

Cell Bodies

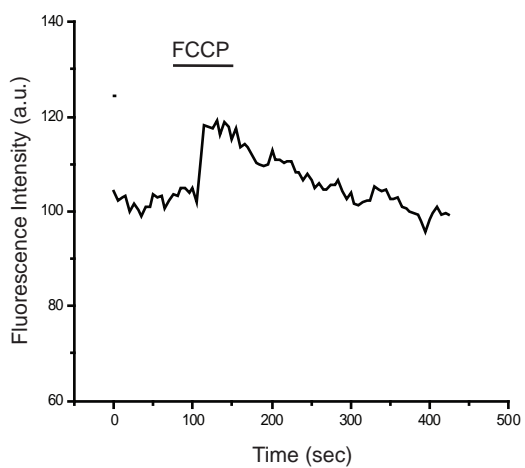


D

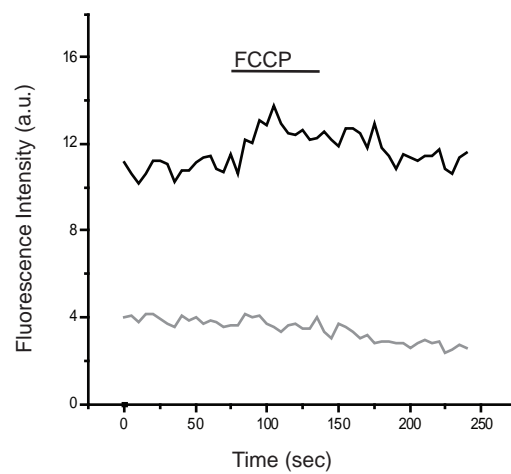
Cell Processes



B

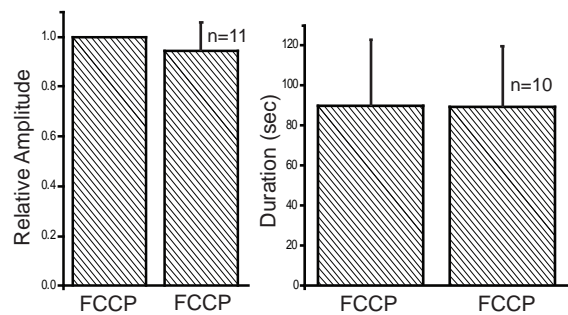


E



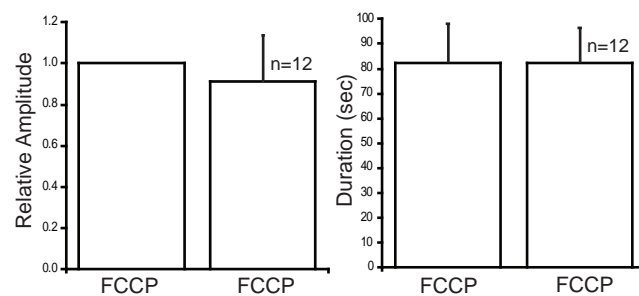
C

Cell Bodies



F

Cell Processes (Near)

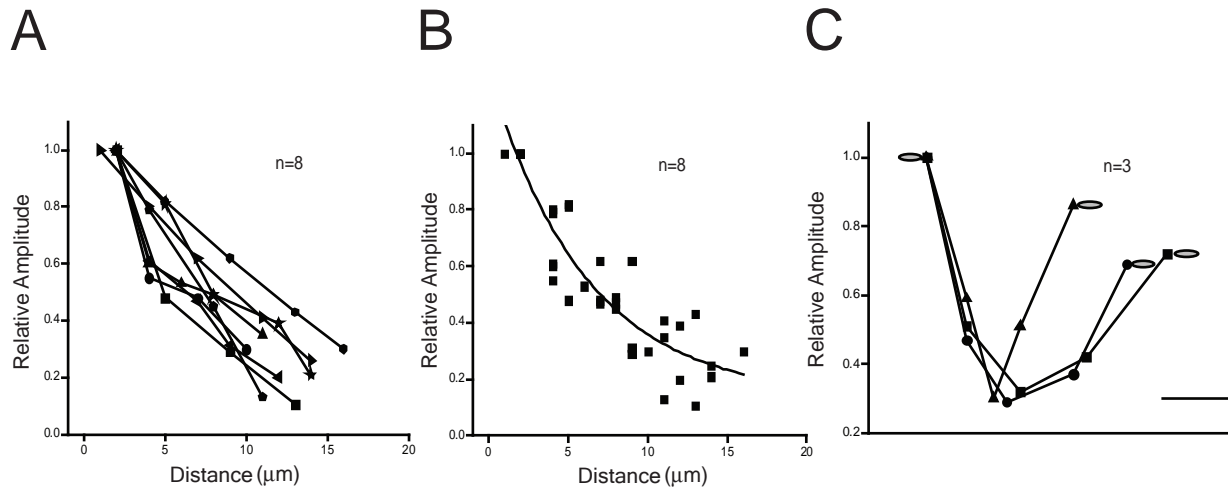


processes  $p=0.947$ ) (measured at half-height, Fig. 3.2C, F) from first FCCP responses suggesting that the source of the  $\text{Ca}^{2+}$  is not depleted in this time frame.

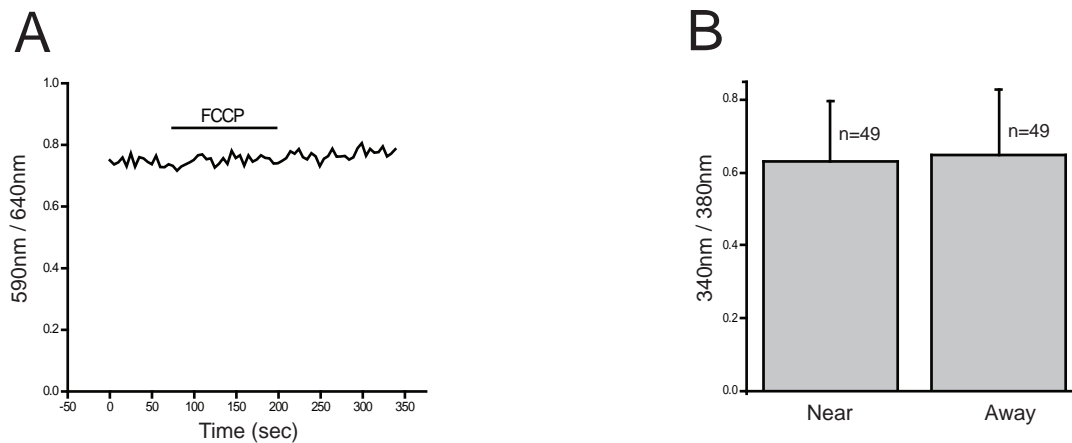
To examine the site of origin of these  $\text{Ca}^{2+}$  elevations, data were collected in processes from regions immediately adjacent to mitochondria as well as regions more than 10  $\mu\text{m}$  away from the nearest mitochondrion. We consistently observed that FCCP produced much smaller  $\text{Ca}^{2+}$  elevations at distant sites (Fig. 3.3). By collecting fluorescence intensity data from multiple contiguous regions of 2  $\mu\text{m}$  in length, we determined that the relative amplitudes of FCCP-dependent  $\text{Ca}^{2+}$  elevations decreased with distance from mitochondria (Fig. 3.3 A, B). If measurements were taken between two mitochondria, the amplitude of the FCCP-dependent  $\text{Ca}^{2+}$  elevation would begin to increase again as measurements were taken closer to the second mitochondrion (Fig. 3.3 C). The dependence of FCCP-dependent  $\text{Ca}^{2+}$  elevations on their proximity to mitochondria supports the idea that these  $\text{Ca}^{2+}$  elevations are mitochondria-dependent. One concern, however, is that a local, FCCP-dependent change in pH alters cytosolic  $\text{Ca}^{2+}$  concentration. To address this and the possible effects of FCCP in general we loaded cells with the pH indicator SNARF-1 AM and monitored pH during FCCP applications. As shown in Figure 3.4, FCCP produced little or no fluctuations in cytosolic pH (Fig. 3.4A), arguing against a secondary effect of pH.

It was consistently observed that resting cytosolic  $\text{Ca}^{2+}$  levels appeared to be higher in regions immediately adjacent to mitochondria and lower at regions more than 10  $\mu\text{m}$  away from mitochondria. This could be a reflection of genuine regional differences in resting cytosolic  $\text{Ca}^{2+}$  levels or an artifact due to the greater thickness of processes adjacent to mitochondria. To resolve this, we loaded the cells with the ratio-metric dye Fura-2 and looked for regional differences in resting cytosolic  $\text{Ca}^{2+}$  concentration. Typically we observed fairly uniform resting  $\text{Ca}^{2+}$  levels throughout processes (Figure 3.4B) indicating that the apparent differences detected





**Figure 3.3 Dependence of FCCP responses on the proximity to mitochondria.** **A**, Relative amplitudes of FCCP-dependent  $\text{Ca}^{2+}$  responses are smaller with the increase in distance from mitochondria. **B**, Trace showing the rate of change of FCCP response amplitude with distance. **C**, Data collected from regions of processes in between two mitochondria in 3 different cells. Ovals indicate mitochondrial position. Relative amplitude of FCCP responses are larger near mitochondria but smaller at distant sites from mitochondria. Scale bar is 5  $\mu\text{m}$ .



**Figure 3.4 Cytosolic pH measurement and ratiometric analysis.** **A**, pH measurement from a representative amacrine cell shows that FCCP (1  $\mu\text{M}$ ) produces no fluctuations in cytosolic pH. **B**, Paired measurements are taken from process of multiple Fura 2-loaded amacrine cells at rest. Regions of processes near and away from mitochondria have relatively uniform resting  $\text{Ca}^{2+}$  levels. **A** contributed by Emily McMains.

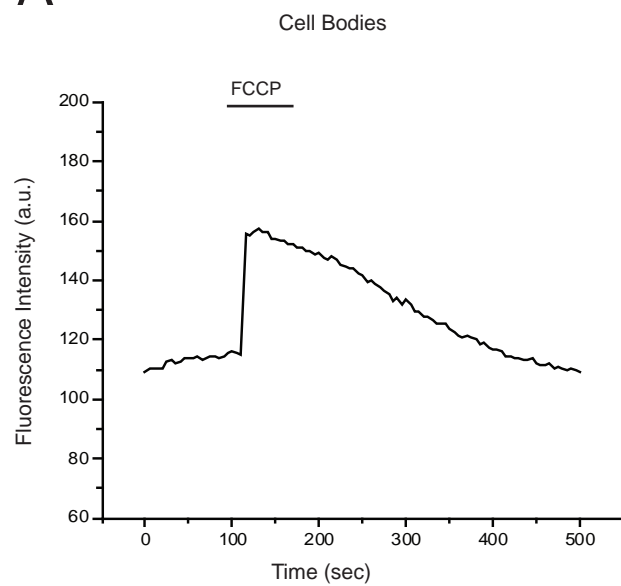
with the single wavelength dye OGB 488 were likely due to regional variation in process thickness. Fortunately, this artifact does not interfere with the evaluation of our data because we either compare the time courses of responses or changes in response amplitudes within the same region of interest over time.

What mechanism underlies the FCCP-dependent  $\text{Ca}^{2+}$  elevations? One possibility is that in amacrine cells, mitochondria store some  $\text{Ca}^{2+}$  at rest. FCCP would shift the balance towards  $\text{Ca}^{2+}$  release via the mitochondrial exchanger producing a local elevation in cytosolic  $\text{Ca}^{2+}$ . Alternatively, it may be that at least some of the  $\text{Ca}^{2+}$  comes from ER. Mitochondria might normally buffer  $\text{Ca}^{2+}$  escaping from the ER via  $\text{IP}_3$  and/or ryanodine receptors (RyRs). This escaping  $\text{Ca}^{2+}$  would be revealed when mitochondrial  $\text{Ca}^{2+}$  uptake is inhibited with FCCP. Another possibility is that the  $\text{Ca}^{2+}$  originating from mitochondria can stimulate ryanodine receptors on the ER membrane via  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR). To explore these possibilities, we ask whether the state of the ER store affects the properties of the FCCP-dependent  $\text{Ca}^{2+}$  elevations.

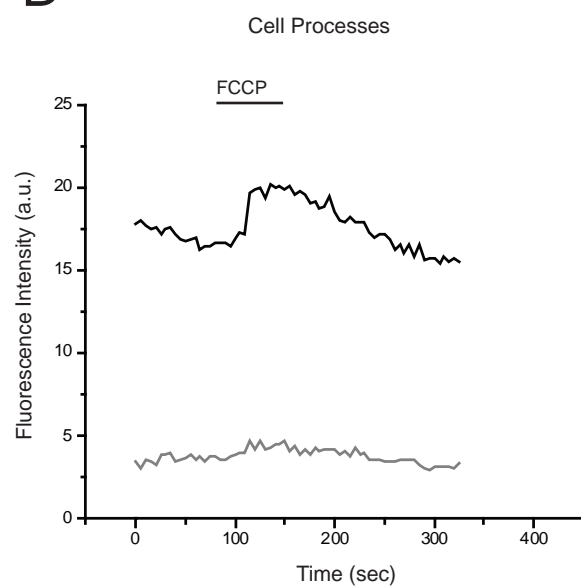
To reduce the  $\text{Ca}^{2+}$  content of the ER, cells were pre-treated with thapsigargin, an irreversible inhibitor of sarcoplasmic-endoplasmic reticulum  $\text{Ca}^{2+}$  pumps (SERCA) pumps (Davidson and Varhol, 1995). A comparison of the time course of FCCP-dependent  $\text{Ca}^{2+}$  elevations between untreated and treated cells indicates that the depletion of  $\text{Ca}^{2+}$  from ER, truncates the response to FCCP (Figure 3.5A, B). A similar reduction in duration was observed in processes near mitochondria in thapsigargin-treated cells (Figure 3.5D, E). Cyclopiazonic acid (CPA) is a reversible inhibitor of SERCA pump activity (Suzuki et al., 1992, Uyama et al., 1992, Demarex et al., 1992). To determine whether CPA would have a similar, but reversible effect on the FCCP-dependent  $\text{Ca}^{2+}$  elevation, FCCP responses were measured before, during

**Figure 3.5. Pre-treatment with thapsigargin alters the duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations.** **A**, FCCP-dependent  $\text{Ca}^{2+}$  elevations are produced in an amacrine cell body. **B**, A representative cell pretreated with thapsigargin (1 $\mu\text{M}$ , 1 hour) produces a relatively brief FCCP-dependent  $\text{Ca}^{2+}$  elevation. **C**, The duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations in thapsigargin-treated cell bodies are significantly different from control cells ( $p < 0.0001$ ). **D**, In processes of a representative cell, FCCP-dependent  $\text{Ca}^{2+}$  elevations are seen in regions next to mitochondria (black trace) are much smaller in regions away from mitochondria (gray trace). **E**, Measurements from a thapsigargin treated cell reveal FCCP-dependent  $\text{Ca}^{2+}$  responses that are typically shorter in duration than control. **F**, The effects of thapsigargin on FCCP-dependent  $\text{Ca}^{2+}$  elevations are also statistically significant in processes ( $p < 0.0001$ ). “a.u.” stands for arbitrary units.

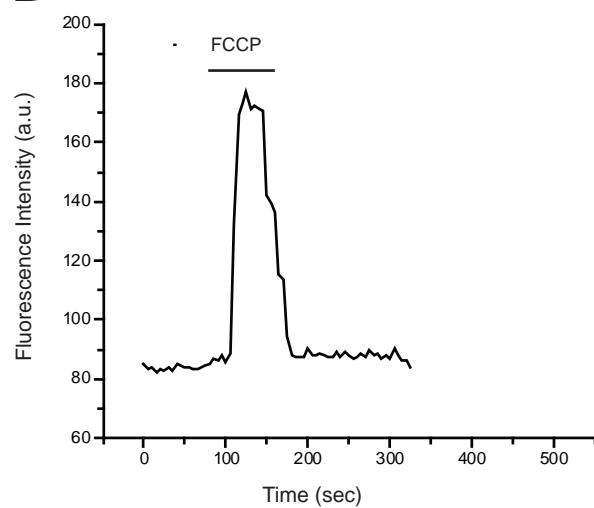
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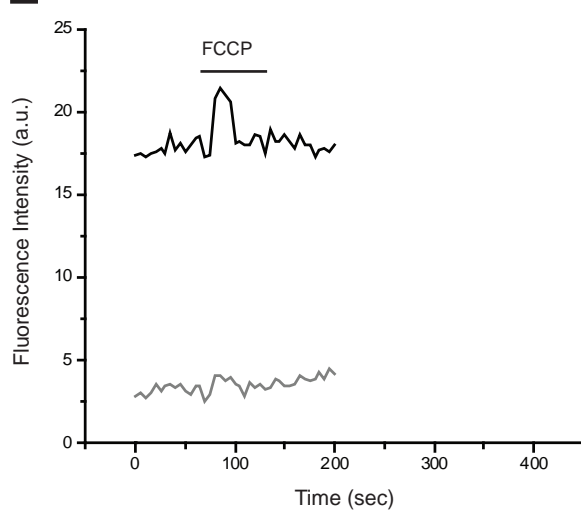
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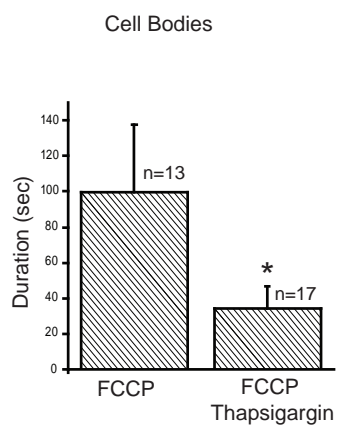
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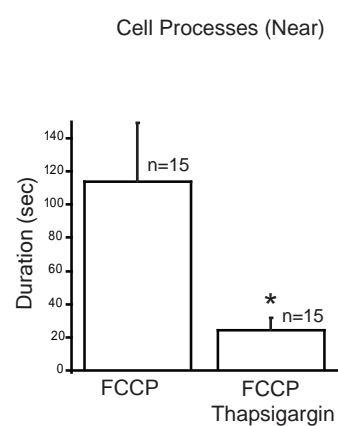
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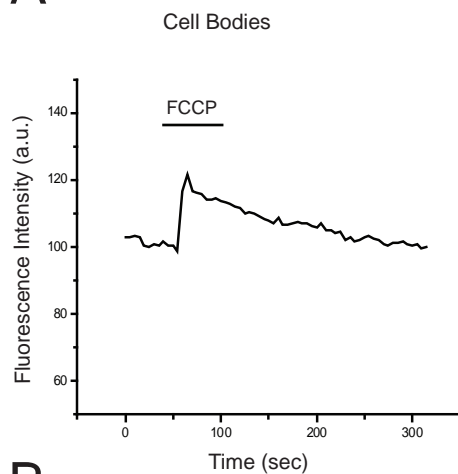
and after exposure (6 min) to CPA. As with thapsigargin, CPA decreased the duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations. The effect of decreased FCCP-dependent  $\text{Ca}^{2+}$  elevation was seen both in cell bodies (Fig. 3.6 A-D) and in processes (Fig. 3.6 E-H). This effect was reversible after a wash of 5 minutes (Fig. 3.6C, D). Reversible reductions in the amplitude of FCCP-dependent  $\text{Ca}^{2+}$  elevations were also observed in both cell bodies and processes.

If the duration of the FCCP-dependent  $\text{Ca}^{2+}$  elevation is dependent upon the  $\text{Ca}^{2+}$  content of the ER, then it is plausible that ER  $\text{Ca}^{2+}$  typically contributes to these responses. The normal routes for  $\text{Ca}^{2+}$  efflux from the ER are the  $\text{IP}_3$  receptor and the ryanodine receptors. These receptor-channel complexes have been previously localized to amacrine cell bodies and processes (Warrier et al., 2005) with an antibody that recognizes the avian homologues for RyR types 1 and 3. To confirm and extend these results we combined antibodies raised against the  $\text{IP}_3\text{R}$  and the RyR Type 3 with an antibody raised against a mitochondria-specific protein. The anti-RyR 3 expression (red) was abundantly seen in the cell bodies and in processes (Fig.3.7A). The high magnification image of the process (Fig.3.7A) showed the punctate labeling pattern of anti-RyR 3 (Fig.3.7D). The distribution of mitochondria (green) was seen in the cell bodies and also in the cell processes (Fig.3.7 B). The localization of mitochondria was demonstrated in the high magnification image of the process (Fig.3.7E). The overlay image indicated that RyRs are localized adjacent to mitochondria (Fig. 3.7C, F). Some amount of co-localization was also observed in the process as indicated in yellow in the overlays (Fig. 3.7F, arrows). The  $\text{IP}_3$  receptors expression (red) was observed in the cell bodies and in the cell processes (Fig.3.7G). The labeling pattern of  $\text{IP}_3$  receptors was observed in the high magnification image (Fig.3.7J).

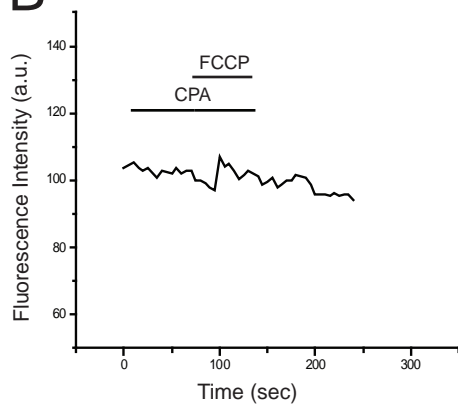
The localization of mitochondria (green) was observed in the cell bodies as well as in the process (Fig.3.7 H, K). The overlay image indicated that the  $\text{IP}_3$  receptors are also present next to mitochondria (Fig. 3.7I, L, arrows) but have less co-localization than RyRs.

**Figure 3.6. CPA alters the duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations in amacrine cells.**  
**A-D**, Data from cell bodies. **A**, FCCP-dependent  $\text{Ca}^{2+}$  elevation in the cell body of a representative amacrine cell before application of CPA. **B**, FCCP response in the same cell body after a 6 minute exposure to CPA. **C**, CPA-dependent changes are reversible after a 5 minute wash. **D**, CPA reversibly reduces the duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations in cell bodies ( $p < 0.0001$ ). **E-H**, Data from processes. **E**, FCCP-dependent  $\text{Ca}^{2+}$  elevations are seen only in regions next to mitochondria (black trace) and not in regions away from mitochondria (gray trace). **F**, FCCP-dependent  $\text{Ca}^{2+}$  elevations are much shorter in duration after application of CPA in processes near mitochondria. **G**, The CPA-dependent changes are reversible in the processes similar to that in the cell bodies. **H**, CPA-dependent effects on the cell processes near mitochondria are statistically significant ( $p = 0.0002$ ). “a.u.” stands for arbitrary units.

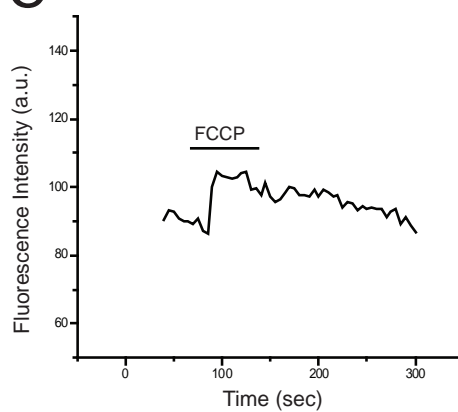
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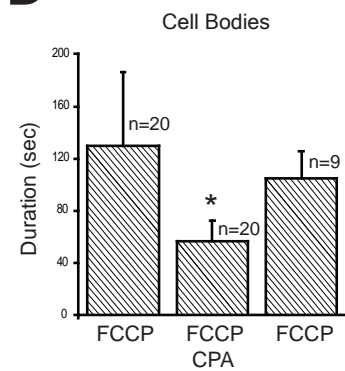
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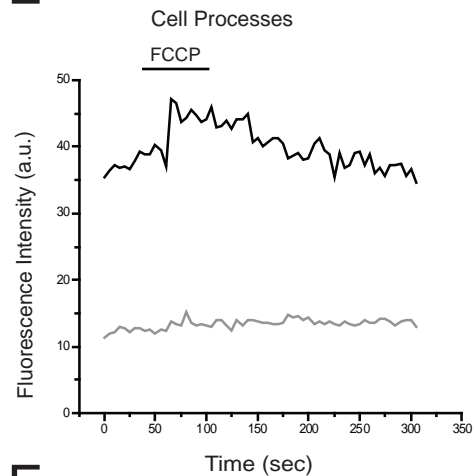
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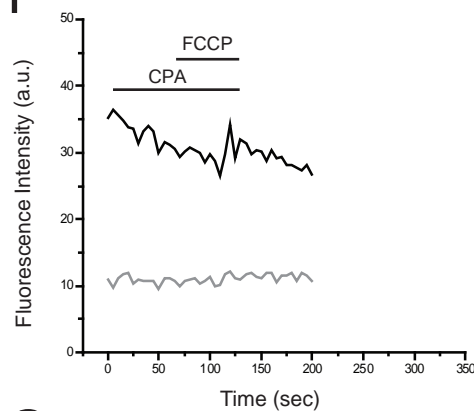
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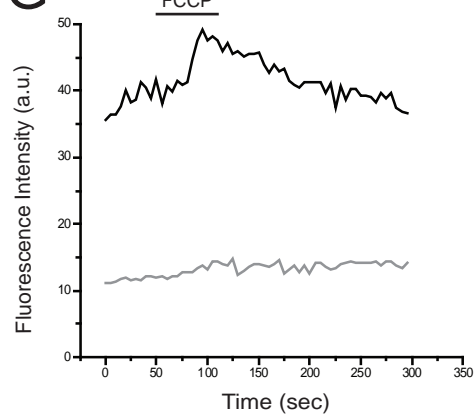
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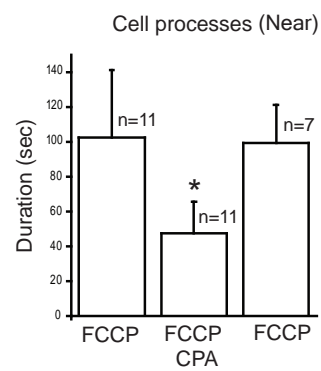
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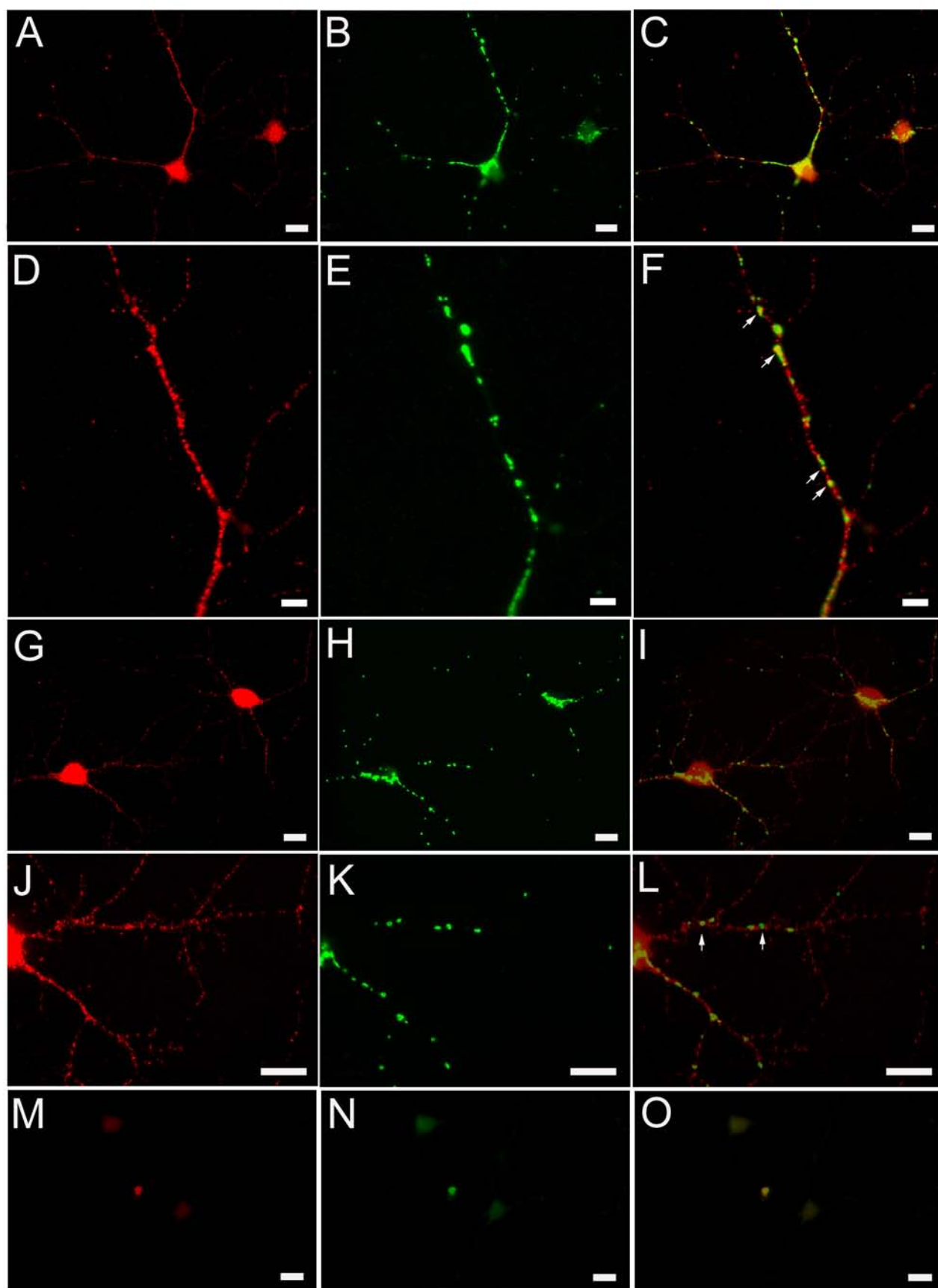


H



**Figure 3.7. Both ryanodine receptors and IP<sub>3</sub> receptors are expressed in amacrine cells.** **A**, Ryanodine receptors (red) are expressed in cell bodies and also in cell processes. **B**, Mitochondrial antibody (4C7/2E4) labeling (green) is seen in the cell bodies and in the cell processes. **C**, The overlay image shows that mitochondria are localized adjacent to ryanodine receptors. **D**, The zoomed in image of the process shows the abundant expression of ryanodine receptors. **E**, The distribution of mitochondria is shown here in the same process. **F**, The overlay image shows the localization of mitochondria next to ryanodine receptors along with some co-localization (arrows). **G**, IP<sub>3</sub> receptors are expressed (red) in the cell bodies and also in the cell processes. **H**, The localization of mitochondria (green) is seen in the cell bodies and in the cell processes. **I**, Overlay image show that the mitochondria are localized next to the IP<sub>3</sub> receptors. **J**, The zoomed in image of the process demonstrates the expression of IP<sub>3</sub> receptors. **K**, The distribution of mitochondria in the processes is shown here in this zoomed in image. **L**, The overlay image of the two indicates the presence of IP<sub>3</sub> receptors primarily adjacent to mitochondria (arrows). **M-O**, Control cells received both secondary antibodies but no primary antibodies. Scale bars are 10  $\mu$ m. **A-O** contributed by Emily McMains.

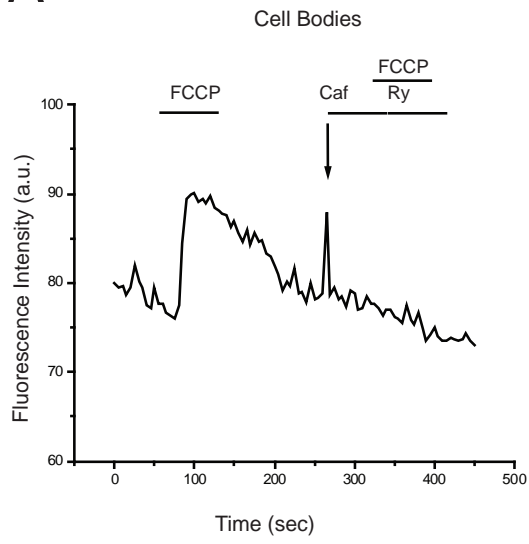




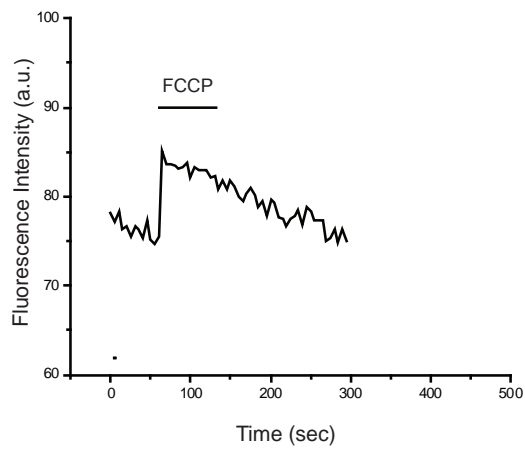
Because amacrine cells apparently express both RyR and IP<sub>3</sub>R, it is possible that either or both receptors participate in FCCP-induced Ca<sup>2+</sup> elevations. To evaluate the role of RyR, the effects of blocking concentrations of ryanodine (20 μM) were examined. The ryanodine application was preceded by a brief pulse of caffeine to confirm the presence of a ryanodine-sensitive Ca<sup>2+</sup> store (Vallot et al., 2001). In the presence of ryanodine, the FCCP-dependent Ca<sup>2+</sup> elevation was completely eliminated in most cells. On average, the response was reduced by ~97%. The effects of ryanodine were similar in cell bodies (Figure 3.8A) and in processes near mitochondria (Figure 3.8D). The effects were reversible after a wash of 5 minutes in both the cell bodies (Figure 3.8B) and in processes near mitochondria (Figure 3.8E). Similar results were obtained in experiments where the caffeine pulse was omitted (not shown). Within-cell comparisons of response amplitudes were made instead of duration because the responses in RyR were either very small or nonexistent. Similar results (data not shown) were seen when cells were treated with dantrolene (20 μM), a reversible inhibitor specific for RyRs 1 and 3 (Zhao et al., 2001). The effectiveness of ryanodine in blocking FCCP-dependent Ca<sup>2+</sup> elevations suggest that RyR but not IP<sub>3</sub>R are involved. Ideally, one would like to directly test for IP<sub>3</sub>R involvement, however the lack of specific IP<sub>3</sub>R inhibitors makes this difficult. Experiments with 2-aminoethoxydiphenyl borate (2 APB) decreased the durations of Ca<sup>2+</sup> elevation by about 50% (data not shown). The known effects of APB on store-operated Ca<sup>2+</sup> entry (SOC) (Bose and Thomas, 2006; Bootman et al., 2002) and also sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) Ca<sup>2+</sup> pumps (Bilmen et al., 2002), however, make these experiments difficult to interpret. Nonetheless, the dramatic effect of ryanodine (and dantrolene) on the FCCP-dependent Ca<sup>2+</sup> elevations indicates that the vast majority of the Ca<sup>2+</sup> is coming from the ER via RyRs. The dependence of the response on the proximity to mitochondria,

**Figure 3.8. Ryanodine blocks FCCP-dependent  $\text{Ca}^{2+}$  elevations.** **A**, FCCP-dependent responses are eliminated in the cell bodies after a 10 sec exposure of caffeine (arrow) followed by a 1 minute exposure to ryanodine. **B**, The ryanodine-dependent effects are reversible after a 5 minute wash. **C**, FCCP-dependent responses after application of ryanodine are significantly reduced ( $p < 0.0001$ ) and the effects are reversed after the wash. **D**, Similar effects are seen in regions of processes next to mitochondria (black trace) where the FCCP-dependent responses are completely eliminated after application ryanodine. Responses are not seen in regions away from mitochondria (gray trace). **E**, The ryanodine-dependent responses recover after the wash in the processes, adjacent to mitochondria (black trace). **F**, Blockade of RyRs significantly reduces the amplitude of FCCP-dependent  $\text{Ca}^{2+}$  elevations ( $p < 0.0001$ ). “a.u.” stands for arbitrary units.

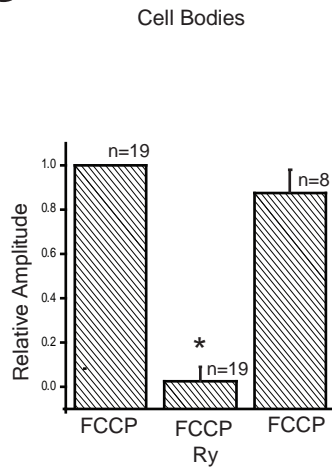
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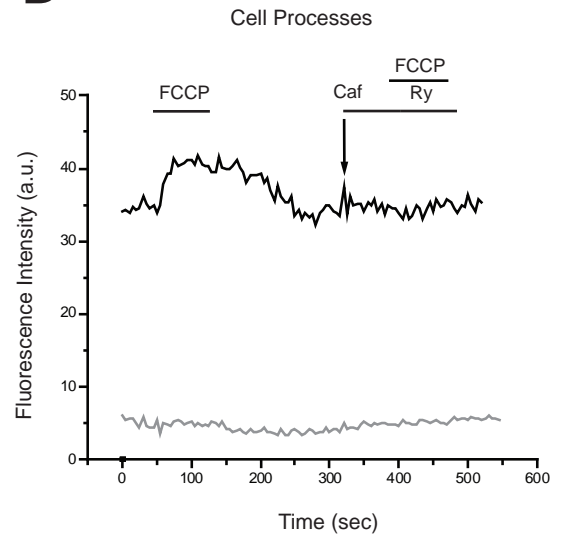
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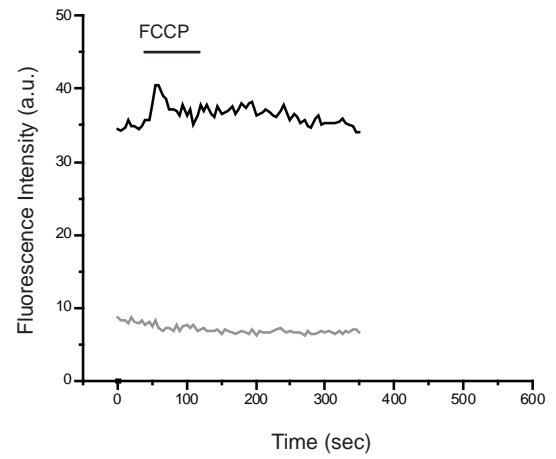
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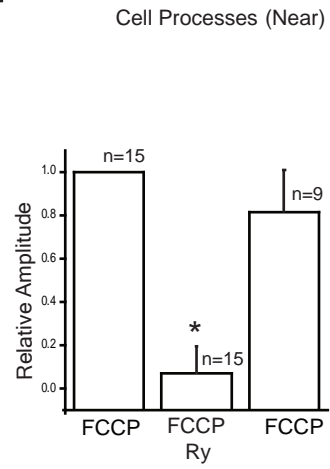
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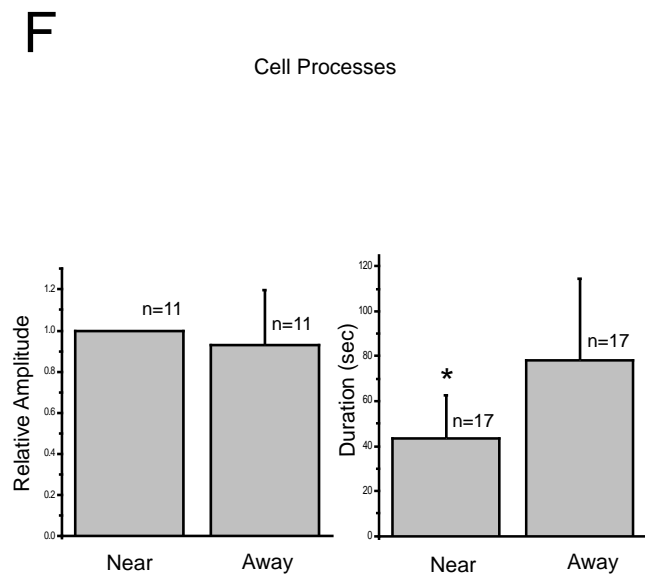
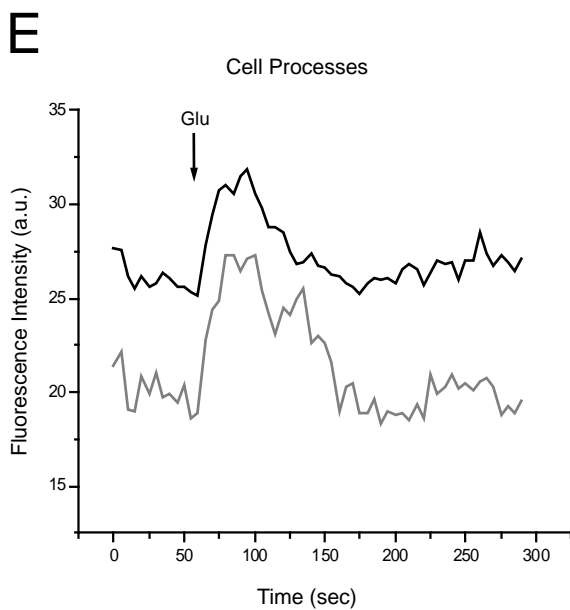
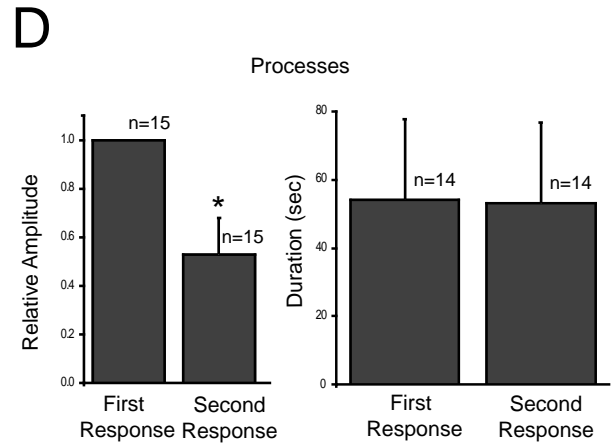
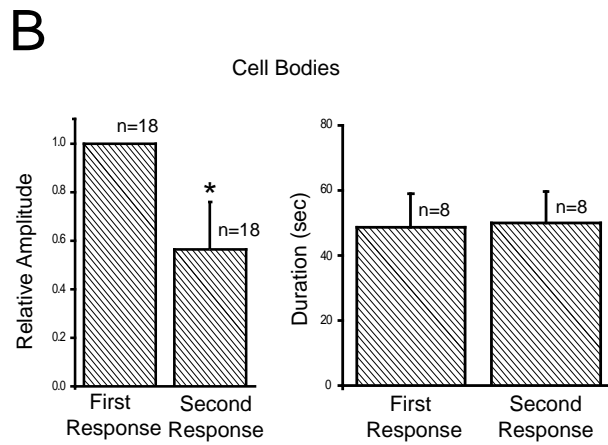
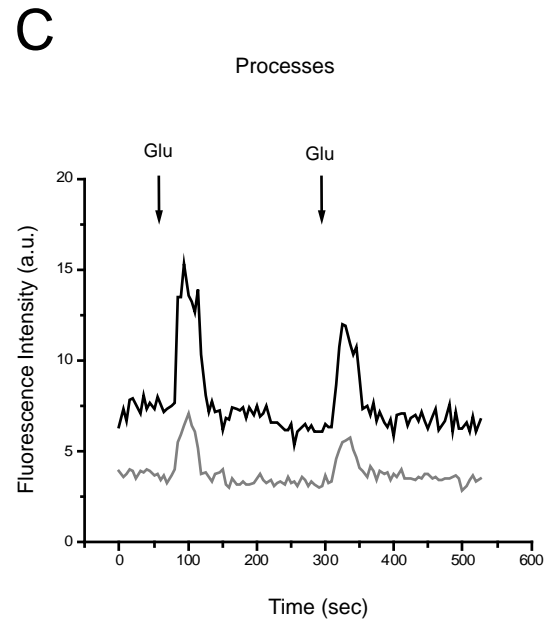
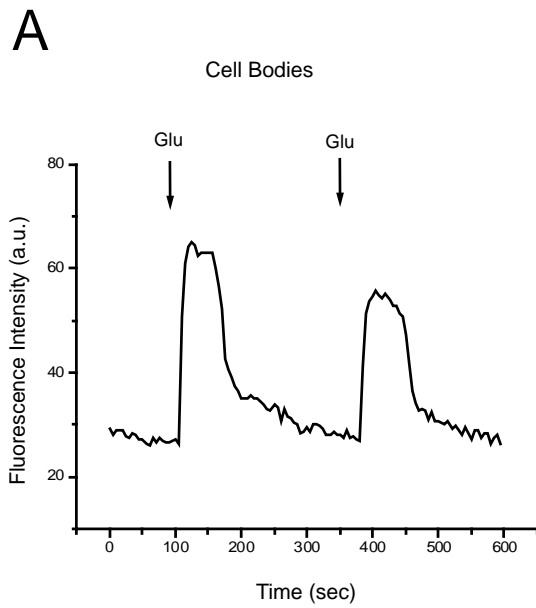
however, suggests that local interactions between RyR and mitochondria are required to generate the response.

### **Glutamate-dependent Calcium Elevations in Cell Bodies and Neuronal Processes**

The results of the previous experiments point to a local interaction between mitochondria and the ER via ryanodine receptors that is likely to contribute to shaping the properties of  $\text{Ca}^{2+}$  elevations in a geographically non-uniform way. This raises the possibility that  $\text{Ca}^{2+}$  influx might also be affected by mitochondria in a localized fashion. To examine this, we have stimulated cells with their endogenous excitatory agonist, glutamate.

Subsequent experiments will involve comparing two glutamate-dependent  $\text{Ca}^{2+}$  elevations elicited under different conditions in the same cell. Therefore, it was important to establish the normal variation between first and second glutamate responses under control conditions. Typically, a reduction in amplitude was observed between the first and second glutamate applications however the time courses of the two responses were not significantly different (Figure 3.9A, B, D). These cells are known to express both AMPA and NMDA ionotropic glutamate receptors (Huba and Hofmann, 1992; Hoffpauir and Gleason, unpublished observations) as well as group 1 metabotropic glutamate receptors (Kreimborg et al., 2001; Sosa, et al., 2002; Hoffpauir and Gleason, 2002). Group 1 metabotropic glutamate receptors are linked to activation of the  $\text{IP}_3$  pathway and subsequent elevations in cytosolic  $\text{Ca}^{2+}$  elevations (Sosa et al., 2002). Current clamp recordings of voltage responses to glutamate application demonstrate that the depolarization produced was sufficient to activate a fraction of L-type  $\text{Ca}^{2+}$  channels (not shown), the predominant channel type expressed by these cells (Gleason, Borges and Wilson, 1994). Thus, glutamate-dependent  $\text{Ca}^{2+}$  elevations can involve activation of ionotropic glutamate receptors, metabotropic glutamate receptors, L-type voltage-gated  $\text{Ca}^{2+}$  channels.

**Figure 3.9. Glutamate-dependent calcium elevations in amacrine cells.** **A**, Second glutamate response is smaller than the first glutamate response in a representative amacrine cell body. **B**, Reduction in relative amplitude between the first and second response is statistically significant in cell bodies ( $p < 0.0001$ ). However, the time course of these responses are not significantly different ( $p = 0.384$ ). **C**, Second application of glutamate generally produces smaller responses in processes as well. **D**, Relative amplitudes of these responses are significantly different in processes ( $p < 0.0001$ ) but the duration of these responses are not different ( $p = 0.214$ ). **E**, Data collected from region of process near a mitochondrion (black trace) and a region of the same process but  $>10 \mu\text{m}$  away from mitochondrion (gray trace). **F**, Within-process comparisons reveal a significant difference in the duration of the glutamate response ( $p < 0.0001$ ) at two different locations in the same process but no difference is seen in the relative amplitude ( $p = 0.405$ ) of these responses. “a.u.” stands for arbitrary units.



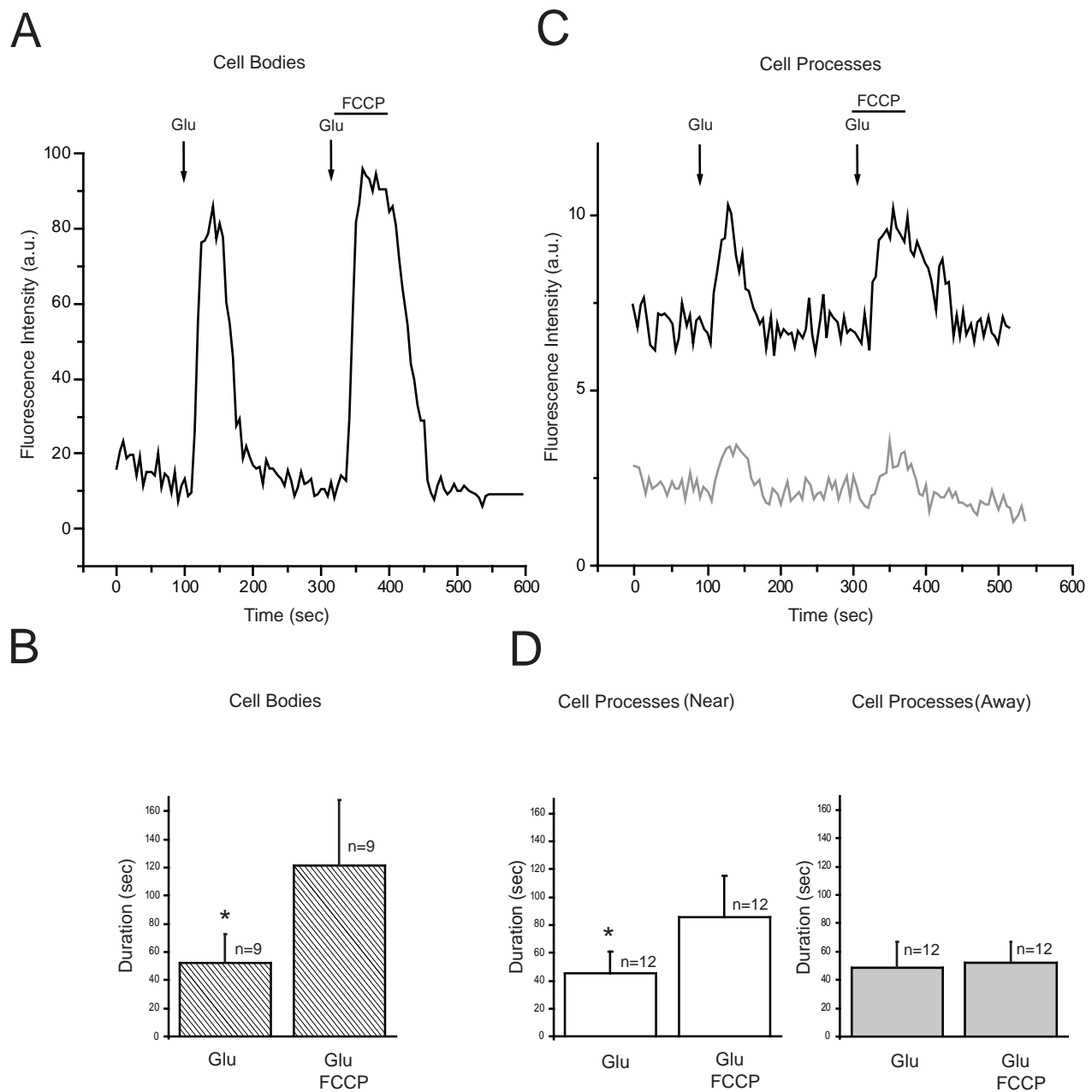
Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release may also contribute to these glutamate-dependent Ca<sup>2+</sup> elevations (Hurtado et al., 2002).

To begin to look for the effects of mitochondrial Ca<sup>2+</sup> transport on glutamate-dependent Ca<sup>2+</sup> elevations in processes, Ca<sup>2+</sup> imaging experiments were performed on cells loaded with MitoTracker Red and OGB 488. OGB fluorescence intensity data were collected from regions in processes adjacent to mitochondria and regions >10 µm away from mitochondria. Within-process comparisons revealed a significant difference in the duration of the responses at the two different locations (Fig. 3.9E, F). Glutamate-dependent Ca<sup>2+</sup> elevations measured next to mitochondria were consistently shorter in duration than those measured > 10 µm away from mitochondria. This result is consistent with a scenario where, in processes, the effects of mitochondrial Ca<sup>2+</sup> transport are spatially limited.

To confirm that these local effects are due to mitochondrial Ca<sup>2+</sup> transport, FCCP was employed to disrupt Ca<sup>2+</sup> uptake via the uniporter (Friel and Tsien, 1994; Babcock and Hille, 1998) and CGP37157 was used to block mNCX function (Cox et al., 1993).

Using FCCP to block mitochondrial Ca<sup>2+</sup> uptake, glutamate-dependent Ca<sup>2+</sup> elevations were measured in cell bodies and regions of primary processes that were situated immediately adjacent to mitochondria and regions at least 10 µm away from any mitochondrion. In cell bodies, glutamate delivered in the presence of FCCP produced Ca<sup>2+</sup> elevations that were more prolonged (measured as duration at half-height) than those elicited in glutamate alone (Fig. 3.10A, B). The same effect could be observed in processes but only when data were gathered from regions immediately adjacent to mitochondria (Fig. 3.10C). The time course of glutamate-dependent Ca<sup>2+</sup> elevations >10 µm away from mitochondria were unaffected by disruption in mitochondrial Ca<sup>2+</sup> uptake (Fig. 3.10D).





**Figure 3.10. Glutamate-dependent  $\text{Ca}^{2+}$  elevations are locally buffered by mitochondria.**

**A**, Recording from a representative amacrine cell body. Glutamate (8 sec application) responses obtained in the presence of FCCP are shown to be prolonged when compared to control. **B**, On average, glutamate-dependent  $\text{Ca}^{2+}$  elevations are significantly ( $p=0.0007$ ) prolonged in the presence of FCCP. **C**, FCCP increases the duration of glutamate-dependent  $\text{Ca}^{2+}$  elevations in cell process near a mitochondrion (black trace) but not in region 10  $\mu\text{m}$  away from any mitochondria (gray trace). **D**, In processes, the duration of FCCP-dependent  $\text{Ca}^{2+}$  elevations are significantly prolonged near mitochondria ( $p=0.0003$ ) but not away from mitochondria ( $p=0.065$ ). “a.u.” stands for arbitrary units.

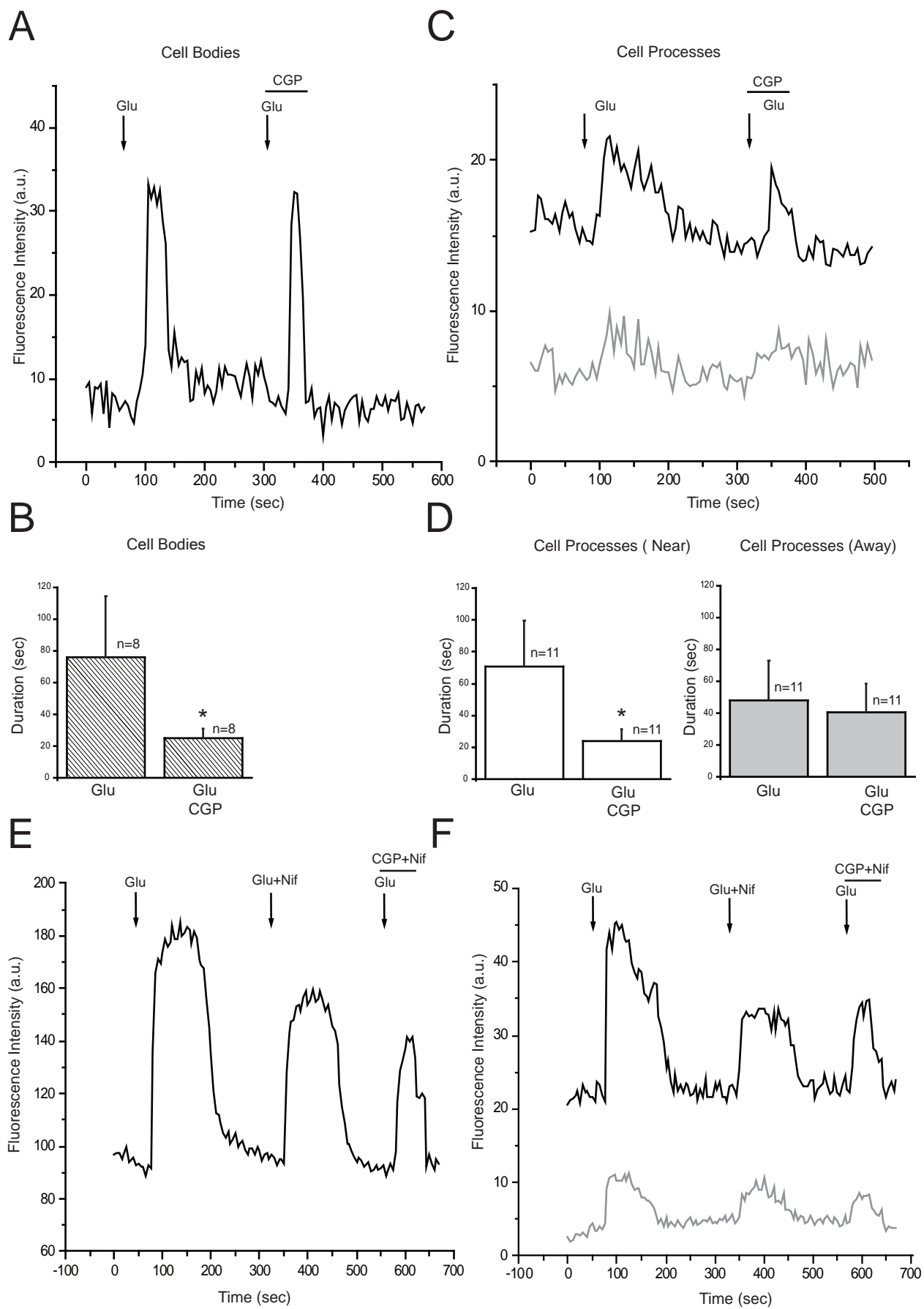
The role of the mNCX in setting the time frame of  $\text{Ca}^{2+}$  in cell bodies and processes was also examined. As previously reported for depolarization-dependent  $\text{Ca}^{2+}$  elevations (Medler and Gleason 2002), the inhibition of the mNCX with CGP 37157 shortened the duration of glutamate-dependent  $\text{Ca}^{2+}$  elevations in amacrine cell bodies (Fig. 3.11A, B). As seen with FCCP, the effects of CGP were only detectable in data collected from regions in processes adjacent to mitochondria (Fig. 3.11C, D). However, CGP 37157 is also known to inhibit L-type  $\text{Ca}^{2+}$  channels (Baron and Thayer, 1997; White and Reynolds, 1997). The position-specific nature of the effects of CGP37157 implies that under these conditions, the major target of CGP37157 was the mNCX. Nonetheless, these experiments were repeated in the presence of the L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (Stork and Cocks, 1994; Segawa et al., 1999; Eliot and Jamali, 1999; Zanger et al., 1999) to rule out the possibility that the effects observed were due to the known inhibitory effect of this reagent on these channels (Fig. 3.11 E, F). Together, these observations indicate that mitochondria influence the time course of glutamate-dependent  $\text{Ca}^{2+}$  elevations in amacrine cells.

## DISCUSSION

In this chapter, I have investigated the role of the two intracellular organelles, mitochondria and ER that contribute to local cytosolic  $\text{Ca}^{2+}$  signaling in retinal amacrine cells. Here I show that  $\text{Ca}^{2+}$  transport mechanisms present in mitochondria and ER can interact with each other and produce local effects. By selectively disrupting  $\text{Ca}^{2+}$  transport mechanisms in mitochondria and the ER, I have elucidated the relative importance of these mechanisms and the interplay between them.

Using FCCP to perturb the balance of  $\text{Ca}^{2+}$  flux into and out of mitochondria, my data suggest that  $\text{Ca}^{2+}$  exiting mitochondria can trigger  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the ER via activation of RyRs. Previous studies in muscle cells have demonstrated that  $\text{Ca}^{2+}$  exiting RyRs

**Figure 3.11. Local re-release of  $\text{Ca}^{2+}$  from mitochondria (mNCX) extends glutamate-dependent  $\text{Ca}^{2+}$  elevations.** **A**, Application of glutamate (8 sec) and CGP (1 minute) decreases the duration of  $\text{Ca}^{2+}$  elevation in the cell body of a representative amacrine cell. **B**, Reduction in the duration of glutamate-dependent  $\text{Ca}^{2+}$  elevations are statistically significant in cell bodies ( $p=0.0064$ ). **C**, In a representative process, the reduction in duration of glutamate-dependent  $\text{Ca}^{2+}$  elevations is observed in a region adjacent to mitochondria (black trace) and not away from mitochondria (gray trace). **D**, Reduction in duration of glutamate-dependent  $\text{Ca}^{2+}$  elevations in processes are statistically significant near mitochondria ( $p=0.0002$ ) but not in regions away from mitochondria ( $p=0.070$ ). **E, F** Experiments in **A and B** are repeated in a different cell but in the presence of nifedipine (an L-type  $\text{Ca}^{2+}$  channel blocker). Under these conditions, CGP has the same effect indicating that the reduction in response duration is not due to blockade of L-type  $\text{Ca}^{2+}$  channels. “a.u.” stands for arbitrary units.



enter mitochondria (Nassar and Simpson, 2000; Szalai et al., 2000; Sharma 2000), however, the results presented here suggest that the exchange of  $\text{Ca}^{2+}$  signals can occur from mitochondria to RyR on the ER membrane as well. How might this sort of interaction influence cellular function? During signaling,  $\text{Ca}^{2+}$  would rapidly enter mitochondria via the uniporter then be more slowly released via mNCX. If the release of  $\text{Ca}^{2+}$  from mitochondria elicits CICR, then a local domain of protracted, elevated  $\text{Ca}^{2+}$  levels would result. The downstream effects of such interactions would be determined by the location of the mitochondria with synaptically located mitochondria being especially interesting to consider. There is evidence that CICR promotes synaptic transmission in hippocampal neurons (Savic and Sciancalepore, 1998; Emptage et al., 2001) and in vestibular hair cells (Lelli et al., 2003). CICR can also underlie depression of synaptic transmission by promoting the activation of presynaptic BK channels (Beurg et al., 2005). Thus the interaction between mitochondria and RyRs has the potential to introduce an additional layer of complexity into the  $\text{Ca}^{2+}$  dynamics of the presynaptic terminal.

I also provide evidence that mitochondria have localized effects on cytosolic  $\text{Ca}^{2+}$  elevations elicited by glutamate indicating that the distribution of mitochondria in neuronal processes will influence the spatial and temporal pattern of cytosolic  $\text{Ca}^{2+}$  elevations. The determinants of mitochondrial distribution are just beginning to be understood (for review see, Hollenbeck and Saxton, 2005). At the molecular level, some key proteins have been identified that regulate the translocation of mitochondria on the cytoskeletal network. These include the mitochondria-associated protein Milton (Stowers et al., 2002) and the atypical Rho GTPases Miro-1 and -2 (Guo et al., 2005; Fransson et al., 2006). Glutamate itself has been shown to inhibit mitochondrial movement by generating a cytosolic  $\text{Ca}^{2+}$  signal via the activation of NMDA receptors (Rintoul et al., 2003) so it may be that the spatial pattern of excitatory synaptic activity plays a role in determining mitochondrial location in neuronal processes.

In the retina, amacrine cells extend their processes and make multiple synapses in the inner plexiform layer. The close proximity of synaptic inputs and outputs on amacrine cell processes suggests that some regions of the cell could function with independence from others. Based on the physiological properties of the cells, simulations demonstrate independence among processes for cultured rat GABAergic amacrine cells (Koizumi et al., 2005). Previous work indicates that amacrine cell processes in the intact retina (Denk and Detwiler, 1999) and in culture (Azuma et al., 2004) exhibit non-uniform  $\text{Ca}^{2+}$  signals within dendritic segments suggesting that mechanisms are in place to keep incoming, excitatory signals local. The physically discrete nature of mitochondria, as well as the local effects of mitochondrial  $\text{Ca}^{2+}$  transport on signals reported here suggests that mitochondria are good candidates for serving this role. Additional studies on the local effects of mitochondria on amacrine cell synaptic transmission will help to resolve the role of mitochondrial  $\text{Ca}^{2+}$  transport in amacrine cell function.

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## **Chapter 4**

### **Conclusions**

In this dissertation I have explored two avenues that contribute to our understanding of retinal signal processing. Chapter 2 describes the expression pattern of Group I mGluRs at synapses in the chicken retina. Chapter 3 describes the role of mitochondrial  $\text{Ca}^{2+}$  transport in regulating  $\text{Ca}^{2+}$  signals in amacrine cell processes. Although the first study was anatomical and done in the intact chicken retina and the second was a physiological study done on cultured amacrine cells, results of both of these studies are key steps in understanding synaptic function in the retina.

## **METABOTROPIC GLUTAMATE RECEPTORS AND AMACRINE CELLS**

Describing the sub-cellular localization of Group I mGluRs in the retina is an important step in understanding the potential physiological functions of these receptors in the retina. Here, I will focus on the expression of these receptors at amacrine cell synapses. GABAergic amacrine cells receive inputs from bipolar cells in the inner plexiform layer. Bipolar cells release glutamate, an excitatory neurotransmitter and depolarize amacrine cells by activating ionotropic glutamate receptors (Wilson, 2003). In this study, I report that group I mGluRs are also expressed at amacrine cell synapses of the chicken retina. This finding implies that group I mGluRs can influence the function of amacrine cell synapses.

Because the expression patterns of these two receptors are so similar, it is likely that mGluRs 1 and 5 are co-expressed. This provides an opportunity for receptor interactions. It has been previously shown that mGluR 1 activates protein kinase C (Catania et al., 1991) and that mGluR 5 can be desensitized by activation of protein kinase C (Gereau and Heinemann, 1998). Furthermore, experiments from our own lab provide evidence that the mGluRs 1 and 5 expressed by cultured amacrine cells do interact functionally (Kreimborg et al., 2001). Using receptor-selective agonists and  $\text{Ca}^{2+}$  imaging, it was found that co-activation of mGluRs 1 and 5 produced smaller  $\text{Ca}^{2+}$  elevations than activation of mGluR5 alone, suggesting that mGluR1

signaling can have an inhibitory effect on mGluR 5 signaling. This limit on mGluR5-mediated  $\text{Ca}^{2+}$  might play an important protective role when bipolar cells are releasing large amounts of glutamate.

Now that I have established that mGluRs 1 and 5 are expressed at amacrine cell synaptic sites, an important next step will be to address the role of mGluR5-dependent  $\text{Ca}^{2+}$  elevations in regulating synaptic function. Again, results from experiments on cultured amacrine cells provide some clues. Previous work from the Gleason lab has demonstrated that activation of mGluR5 leads to enhancement of currents through both L-type voltage-gated  $\text{Ca}^{2+}$  channels (Sosa and Gleason, 2004) and GABA<sub>A</sub> receptors (Hoffpauir and Gleason, 2002). These observations imply that activation of mGluR5 would amplify activity at amacrine cell synapses because L-type voltage-gated  $\text{Ca}^{2+}$  channels are found presynaptically in amacrine cells (Hoffpauir and Gleason, 2005) and GABA<sub>A</sub> receptors are found postsynaptically (Gleason et al., 1993).

A final point is that although we know that activation of mGluR5 can produce  $\text{Ca}^{2+}$  elevations in cultured GABAergic amacrine cells, we know relatively little about how the  $\text{Ca}^{2+}$  signals generated by activation of these (and other) receptors are regulated. Spatial and temporal patterns of cytosolic  $\text{Ca}^{2+}$  elevations are determined by  $\text{Ca}^{2+}$  transport mechanisms controlling influx to, and efflux from, the cytosol. The relative importance of different  $\text{Ca}^{2+}$  transport mechanisms will depend upon their functional properties, their location in the cell, and their proximity to other sources and sinks for  $\text{Ca}^{2+}$ .

## **MITOCHONDRIA AND CALCIUM SIGNALING IN AMACRINE CELLS**

Chapter 3 focuses on understanding the role that mitochondria play in regulating  $\text{Ca}^{2+}$  signaling in amacrine cells. My results indicate that mitochondrial  $\text{Ca}^{2+}$  transport influences multiple aspects of  $\text{Ca}^{2+}$  signaling in amacrine cell bodies and processes. Furthermore, I have evidence that ryanodine receptors on the ER can sense  $\text{Ca}^{2+}$  leaving nearby mitochondria and

respond by releasing  $\text{Ca}^{2+}$  from the ER via CICR. Another important component of Chapter 3 is the demonstration that in processes, mitochondria shape the temporal properties of glutamate-dependent  $\text{Ca}^{2+}$  elevations in a spatially restricted way. These results imply that, in the retina, mitochondria have the potential to contribute to the non-uniform  $\text{Ca}^{2+}$  observed in amacrine cell processes in the intact retina (Denk and Detwiler, 1999).

Although I have made progress in understanding the function of mitochondria in regulating intracellular  $\text{Ca}^{2+}$  in amacrine cells, much remains to be explored. This study focused on local release and subsequent uptake of  $\text{Ca}^{2+}$  within amacrine cells. It will be important to understand how this local release of  $\text{Ca}^{2+}$  is associated with synaptic transmission in amacrine cells. Amacrine cells make synapses onto bipolar, ganglion and (most commonly) other amacrine cells. How does mitochondrial  $\text{Ca}^{2+}$  transport affect synaptic output from amacrine cells? Electrophysiological experiments can help us by measuring  $\text{Ca}^{2+}$ -dependent synaptic transmission between amacrine cells. One of the major reasons that the Gleason lab uses primary cultures derived from the chick retina is because the identified amacrine cells form functional GABAergic synapses with one another in these cultures (Gleason et al., 1993). These synapses are highly relevant to synaptic transmission in the inner plexiform layer of the retina because it is estimated that GABAergic amacrine cell synapses comprise over 90% of the synapses in this layer (Marc and Liu, 2000). Recordings of evoked or spontaneous postsynaptic currents give the experimenter a sensitive probe into the  $\text{Ca}^{2+}$ -dependent process of presynaptic neurotransmitter release. Under these circumstances, altering the function of mitochondrial  $\text{Ca}^{2+}$  transport will help to elucidate the role of mitochondria at amacrine cell synapses.

It is well documented in a variety of cell types that mitochondria and ER are physically associated (Rizzuto et al., 1998). This structural proximity of the two organelles has proven to be important in cytosolic  $\text{Ca}^{2+}$  regulation (Rizzuto et al., 2004). It will be informative to extend

my observations on calcium signaling in cultured amacrine cells to intact retinal tissue. One approach would be to employ transmission electron microscopy to establish the sub-cellular localization of mitochondria in amacrine cell processes as well as in other retinal cell types.

It will also be important to more precisely identify the molecular players involved. Here, I demonstrated the expression of ryanodine receptors by performing immunolabeling experiments with anti-ryanodine receptor 3. It has already been established that alpha and beta isoforms present in the chick skeletal muscle are homologues of mammalian ryanodine receptor 1 and 3 (Ottini et al., 1996). Because these receptors have distinctive properties, it will be important to know which isoforms of ryanodine receptors are present in amacrine cells and involved in  $\text{Ca}^{2+}$  signaling. In addition to ryanodine receptors, amacrine cells also express  $\text{IP}_3$  receptors on the ER membrane. Studies indicate that  $\text{IP}_3$  receptors are involved in both spontaneous and evoked transmitter release in amacrine cells (Warrier et al., 2005) however, the factors responsible for allowing release of  $\text{Ca}^{2+}$  from  $\text{IP}_3$  receptors remain to be explored. Thus, determination of the spatial organization of mitochondria and ER as well as a further identification of the molecular components involved will enable us to expand our knowledge of  $\text{Ca}^{2+}$ -dependent signaling in amacrine cells.

## **LOCAL CALCIUM SIGNALING**

What have we learned about the spatial aspects of  $\text{Ca}^{2+}$  signaling in other cells? It has been established that the spatially restricted or “local”  $\text{Ca}^{2+}$  signals occur in a variety of cell types (Bootman et al., 2001). The term “local” is used to describe a spatial limit to cytosolic  $\text{Ca}^{2+}$  responses within a cell ranging from ~10nm to several micrometers depending on the type of signaling and the cell types. Non-excitable cells demonstrate a special type of local signaling known as “ $\text{Ca}^{2+}$  puffs” (Bootman, 1996). The spatial spread of these puffs is ~6  $\mu\text{m}$  and the temporal limit is ~1 second. Puffs were first seen in *Xenopus* oocytes (Yao et al., 1995).  $\text{Ca}^{2+}$



puffs are also observed in HeLa cells (Bootman et al., 1997a) and endothelial cells (Huser and Blatter, 1997). There is also evidence of local  $\text{Ca}^{2+}$  signals in excitable cells such as cardiac myocytes (Cheng et al., 1993), skeletal muscle (Tsugorka et al., 1995) and smooth muscle (Jaggar et al., 2000). This type of  $\text{Ca}^{2+}$  signal is called a “ $\text{Ca}^{2+}$  spark”. The sparks in cardiac muscle result from spontaneous opening of  $\text{Ca}^{2+}$  release channels (RyRs) on sarcoplasmic reticulum (Cheng et al., 1993). Sparks are also seen in skeletal muscle where they participate in contraction of muscles (Tsugorka et al., 1995).

Local  $\text{Ca}^{2+}$  signaling also occurs in neurons. Evidence for this is seen at both pre- and post-synaptic sites (Augustine et al., 2003). Presynaptically, these are known either as microdomains or nanodomains of  $\text{Ca}^{2+}$  signals from voltage-gated  $\text{Ca}^{2+}$  channels. At postsynaptic sites, local  $\text{Ca}^{2+}$  signaling occurs through voltage-gated  $\text{Ca}^{2+}$  channels, neurotransmitter-gated channels and release from internal stores (Augustine et al., 2003). Another example of neuronal local  $\text{Ca}^{2+}$  signaling occurs in dendritic spine. Dendritic spines are extensions from the surface of some dendrites that have post synaptic densities. There is evidence that the spine neck can compartmentalize  $\text{Ca}^{2+}$  signaling from the rest of the dendrite and thereby spatially restrict the  $\text{Ca}^{2+}$  elevation (Sabatini et al., 2001, Yuste et al., 1999).  $\text{Ca}^{2+}$  signals in spines arise from voltage-gated  $\text{Ca}^{2+}$  channels, influx through receptors or from intracellular stores.  $\text{Ca}^{2+}$  signals in the spine are spatially restricted and these are short-lived (Berridge, 1998). These  $\text{Ca}^{2+}$  signals are local in that they do not propagate through the dendritic arbor. Instead, they terminate abruptly at dendritic branch points (Finch and Augustine, 1998).

Amacrine cells in the retina are diverse neurons based on their morphology, size (MacNeil and Masland, 1998), and function (Vaney, 2003). It is already known that amacrine cell synaptic signaling occurs out in the processes and that synaptic inputs and outputs in these cells can be present side by side in the processes (Dowling and Boycott, 1966). This

arrangement suggests that signaling in these cells can be highly localized. One well studied type of amacrine cell is the starburst amacrine cell. Starburst amacrine cells have circular dendritic fields (Rodieck, 1989) and are thought to establish directional selectivity for retinal ganglion cells (Yoshida et al., 2001). Recent studies have demonstrated that amacrine cell processes exhibit direction selectivity locally in dendritic branches (Euler et al., 2002). The asymmetry of synaptic inputs and outputs of these starburst amacrine cells enables them to stimulate ganglion cells only when the visual stimulus moves in the preferred direction (He and Masland, 1997, Euler and Denk, 2001). Directional selectivity of starburst amacrine cells can be observed in their distal dendrites. Two-photon optical recordings of intracellular  $\text{Ca}^{2+}$  demonstrated that individual dendritic branches of the starburst amacrine cells respond independently to moving light stimuli and that these directionally selective signals are not transmitted to the amacrine cell body (Euler et al., 2002).

Clearly, the starburst amacrine cell has been designed by evolution to perform a very fundamental operation in the vertebrate retina: the detection of motion. What about the other twenty plus morphological types of amacrine cells? The role of a few other types of amacrine cells is known (AII amacrine cells, dopaminergic amacrine cells, for example) but the role of most remains unknown. Further, physiological studies on amacrine cells in the intact retina will be required to understand the full range of amacrine cell function. These studies, combined with a better understanding of the subcellular mechanisms that determine the signaling capabilities of amacrine cells will be critical to understanding how visual information is processed in the inner retina.

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Madhumita Sen was born in Kolkata, West Bengal, India, on December 1, 1974. She received her primary education in Loreto Day School, Kolkata, and secondary education in Loreto House, Kolkata. She was awarded the Honors Bachelor degree (B.Sc.) with a 1<sup>st</sup> class in zoology from University of Kolkata. She received National Scholarship Award in 1996. She was awarded a 1<sup>st</sup> class in zoology with a special paper in hematology in the master's program (M.Sc.). She enrolled in the doctoral program in the Department of Biological Sciences at Louisiana State University in August 2000 under the guidance of Dr. Evanna Gleason. She will complete all the requirements for the Doctor of Philosophy in July 2006.